D1

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00 — A2

(11) International Publication Number: **WO 99/12576**

(43) International Publication Date: 18 March 1999 (18.03.99)

(21) International Application Number: PCT/US98/19267

(22) International Filing Date: 11 September 1998 (11.09.98)

(30) Priority Data:
60/058,511 11 September 1997 (11.09.97) US

(71) Applicant *(for all designated States except US)*: MUSC FOUNDATION FOR RESEARCH DEVELOPMENT [US/US]; Suite 305, 141 Musc Complex, Cannon Park Place, Charleston, SC 29425 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CHAO, Lee [US/US]; 1011 Scottland Drive, Mt. Pleasant, SC 29464 (US). CHAO, Julie [US/US]; 1011 Scottland Drive, Mt. Pleasant, SC 29464 (US).

(74) Agents: MILLER, Mary, L. et al.; Needle & Rosenberg, P.C., 127 Peachtree Street, N.E., Atlanta, GA 30303 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS AND COMPOSITIONS FOR TREATING CARDIAC AND RENAL DISORDERS WITH ATRIAL NATRIURETIC PEPTIDE AND TISSUE KALLIKREIN GENE THERAPY

(57) Abstract

The present invention provides a method for treating a nonhypertension–associated renal disorder in a subject having a nonhypertension associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating a nonhypertension–associated renal disorder. Also provided is a method of treating a nonhypertension–associated cardiac disorder in a subject having a nonhypertension–associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating the nonhypertension–associated cardiac disorder.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS AND COMPOSITIONS FOR TREATING CARDIAC AND RENAL DISORDERS WITH ATRIAL NATRIURETIC PEPTIDE AND TISSUE KALLIKREIN GENE THERAPY

This invention was made with government support under the National Institutes of Health grant numbers HL 29397 and HL 56686. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods for delivering tissue kallikrein and atrial natriuretic peptide genes to cells via gene therapy mechanisms for the treatment of hypertension-associated and nonhypertension-associated renal and cardiac disorders.

Background Art

Tissue kallikrein is a member of a homologous serine proteinase family which is capable of processing low-molecular-weight kininogen to release the bioactive kinin peptide (1,2). Intact kinins bind to bradykinin B2 receptors and trigger a broad spectrum of biological effects, including smooth muscle contraction and relaxation, electrolyte balance and glucose transport, pain induction, inflammation, increase in vascular permeability, and reduction of blood pressure (2). Extensive clinical studies showed that urinary kallikrein levels are inversely correlated with blood pressure (3-5). Since urinary kallikrein originates from the kidney, the correlation between high blood pressure and reduced urinary kallikrein levels suggests the participation of renal kallikrein in blood pressure homeostasis. Although these clinical studies have implicated a role of renal kallikrein in hypertension, the results are based on random population samples which do not lend themselves to rigorous genetic analyses. A study aimed at identifying genetic factors associated with cardiovascular risks using family pedigrees concluded that a dominant gene expressed as renal or urinary kallikrein may be associated with a reduced risk of hypertension (6). Reduced urinary kallikrein excretion has also been described in a number of genetically hypertensive rats (7-10).

For example, total renal kallikrein content of Dahl salt-sensitive rats was shown to be lower than that of Dahl salt-resistant rats on high, normal, and low salt diets (11).

Atrial natriuretic peptide (ANP) is a 28-amino acid peptide hormone secreted predominantly by atrial cardiomyocytes (12,13). The administration of exogenous ANP results in numerous physiological responses, including a rapid natriuresis and diuresis as well as a reduction in the arterial blood pressure (14-21). ANP has been shown to cause blood pressure reduction in animals and hypertensive human subjects when applied as a bolus injection or in short term infusions (22-28). A genetic defect in ANP production could lead to salt-sensitive hypertension in mice with a disruption of the mouse ANP gene (29).

Short term infusion and repeated administration studies of both TK and ANP have demonstrated that these substances reduce blood pressure in laboratory animals and hypertensive patients, but that the effect lasts only as long as these compounds are present at therapeutic levels, which is a very brief period because these exogenous compounds are very short-lived *in vivo*. Therefore, an effective treatment regimen would require continuous infusion of these substances into individuals. Because of this practical limitation, these compounds have never been commercially developed as viable therapeutic agents. In addition, there are numerous nonhypertension-associated renal (e.g., antibiotic-induced nephrotoxicity) and nonhypertension-associated cardiac disorders for which no successful treatment has been developed.

The present invention overcomes previous limitations in the art by providing methods and compositions for administering TK and ANP via gene therapy mechanisms to individuals for the treatment of both hypertension-associated as well as nonhypertension-associated renal and cardiac disorders which are responsive to elevated amounts of TK and ANP.

SUMMARY OF THE INVENTION

The present invention provides a method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension-associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a nonhypertension-associated renal disorder.

Further provided in the present invention is a method for treating a hypertension-associated renal disorder in a subject having a hypertension-associated renal disorder comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a hypertension-associated renal disorder.

In addition, the present invention provides a method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension associated renal disorder comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating a nonhypertension-associated renal disorder.

Also provided is a method for treating a hypertension-associated renal disorder in a subject having a hypertension associated renal disorder comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating a hypertension-associated renal disorder.

Additionally, the present invention provides a method for treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid

encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a nonhypertension-associated cardiac disorder.

Further provided in the present invention is a method for treating a hypertension-associated cardiac disorder in a subject having a hypertension-associated cardiac disorder comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a hypertension-associated cardiac disorder.

A method is also provided for treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating the nonhypertension-associated cardiac disorder.

Additionally provided is a method for treating a hypertension-associated cardiac disorder in a subject having a hypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating the hypertension-associated cardiac disorder.

The present invention further provides a nucleic acid comprising an isolated nucleic acid encoding tissue kallikrein and an isolated nucleic acid encoding atrial natriuretic peptide as well as a composition comprising a vector comprising a nucleic acid encoding tissue kallikrein and a vector comprising a nucleic acid encoding atrial natriuretic peptide.

Also provided in the present invention is a cell which has been genetically engineered to contain an exogenous nucleic acid encoding tissue kallikrein and an

exogenous nucleic acid encoding atrial natriuretic peptide, as well as the cell of this invention in a pharmaceutically acceptable carrier.

Furthermore, the present invention provides a method for delivering tissue kallikrein and atrial natriuretic peptide to a cell comprising administering to the cell a nucleic acid encoding tissue kallikrein and atrial natriuretic peptide under conditions whereby the nucleic acid is expressed, thereby delivering tissue kallikrein and atrial natriuretic peptide to the cell.

Additionally provided is a method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the nonhypertension-associated renal disorder.

Further provided is a method of treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the nonhypertension-associated cardiac disorder.

A method is also provided herein for treating a hypertension-associated renal disorder in a subject having a hypertension associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the hypertension-associated renal disorder.

Finally provided is a method of treating a hypertension-associated cardiac disorder in a subject having a hypertension associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the hypertension-associated cardiac disorder.

DETAILED DESCRIPTION OF THE INVENTION

As used in the claims, "a" can include multiples.

The present invention provides a method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension-associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a nonhypertension-associated renal disorder.

Further provided in the present invention is a method for treating a hypertension-associated renal disorder in a subject having a hypertension-associated renal disorder comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a hypertension-associated renal disorder.

In addition, the present invention provides a method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension associated renal disorder comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating a nonhypertension-associated renal disorder.

Also provided is a method for treating a hypertension-associated renal disorder in a subject having a hypertension associated renal disorder comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating a hypertension-associated renal disorder.

The cell in the subject in which the nucleic acid encoding tissue kallikrein or atrial natriuretic peptide is expressed to treat a non-hypertension-associated or hypertension-associated renal disorder can be any cell which can take up and express exogenous DNA, including, but not limited to, a heart cell, kidney cell, liver cell, lung cell, adrenal gland cell, endothelial cell, neuronal cell, myoblast and hematopoietic stem cell and is most preferably a kidney cell. In addition, the renal disorder to be treated by administration of a nucleic acid encoding tissue kallikrein can be, but is not limited to, renal injury, nephrotoxicity, nonhypertension-associated or hypertension-associated renal disease, salt-induced renal damage, glomerulosclerotic lesions, tubular injury, drug-induced renal damage, chronic renal failure, acute renal failure, nephrotic syndrome and diabetic nephropathy.

Additionally, the present invention provides a method for treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a nonhypertension-associated cardiac disorder.

Further provided in the present invention is a method for treating a hypertension-associated cardiac disorder in a subject having a hypertension-associated cardiac disorder comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a hypertension-associated cardiac disorder.

A method is also provided for treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating the nonhypertension-associated cardiac disorder.

Additionally provided is a method for treating a hypertension-associated cardiac disorder in a subject having a hypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating the hypertension-associated cardiac disorder.

The cell in the subject in which the nucleic acid encoding tissue kallikrein and/or atrial natriuretic peptide is expressed to treat a nonhypertension-associated or hypertension-associated cardiac disorder can be any cell which can take up and express exogenous DNA, including, but not limited to, a heart cell, kidney cell, liver cell, lung cell, adrenal gland cell, endothelial cell, neuronal cell, myoblast and hematopoietic stem cell and is most preferably a cardiac cell. In addition, the cardiac disorder to be treated by administration of a nucleic acid encoding tissue kallikrein can be, but is not limited to, cardiac hypertrophy, nonhypertension-associated or hypertension-associated cardiac damage, heart failure after cardiac surgery, cardiac injury after myocardial infarction, myocardial ischemia, congestive heart failure, restenosis following angioplasty, cerebravascular disorders such as stroke and occlusive artery diseases and disorders.

The present invention further provides a nucleic acid comprising both an isolated nucleic acid encoding tissue kallikrein and an isolated nucleic acid encoding atrial natriuretic peptide. As used herein, the term "isolated" means a nucleic acid separated or substantially free from at least some of the other components of the naturally occurring organism, for example, the cell structural components commonly found associated with nucleic acids in a cellular environment and/or other nucleic acids. The isolation of nucleic acids can therefore be accomplished by techniques such as cell

lysis followed by phenol plus chloroform extraction, followed by ethanol precipitation of the nucleic acids (30). The nucleic acids of this invention can be isolated from cells according to methods well known in the art for isolating nucleic acids. Alternatively, the nucleic acids of the present invention can be synthesized according to standard protocols well described in the literature for synthesizing nucleic acids.

It is understood that, where desired, modification and changes may be made in the structure of the TK and/or ANP of the present invention and still obtain a protein having like or otherwise desirable characteristics. Such changes may occur in natural isolates or may be synthetically introduced using site-specific mutagenesis, the procedures for which, such as mis-match polymerase chain reaction (PCR), are well known in the art.

For example, certain amino acids may be substituted for other amino acids in a TK and/or ANP protein without appreciable loss of functional activity of the TK and/or ANP. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a TK and/or ANP amino acid sequence (or, of course, the underlying nucleic acid sequence) and nevertheless obtain TK and/or ANP with like properties. It is thus contemplated by the inventors that various changes may be made in the sequence of the TK and/or ANP amino acid sequence (or underlying nucleic acid sequence) without appreciable loss of biological utility or activity and possibly with an increase in such utility or activity.

The present invention further provides a composition comprising a vector comprising a nucleic acid encoding tissue kallikrein and a vector comprising a nucleic acid encoding atrial natriuretic peptide. The composition can be in a pharmaceutically acceptable carrier. The vector can be an expression vector which contains all of the genetic components required for expression of the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide in cells into which the vector has been introduced, as are well known in the art. The expression vector can be a commercial expression vector or it can be constructed in the laboratory according to

standard molecular biology protocols. The expression vector can comprise viral nucleic acid including, but not limited to, adenovirus, retrovirus and or adeno-associated virus nucleic acid. The nucleic acid or vector of this invention can also be in a liposome or a delivery vehicle which can be taken up by a cell via receptor-mediated or other type of endocytosis.

For example, the nucleic acid comprising an isolated nucleic acid encoding tissue kallikrein and an isolated nucleic acid encoding atrial natriuretic peptide can be inserted into an adenoviral nucleic acid according to methods well known in the art and as described in the Examples herein, wherein the nucleic acids of this invention can be packaged in an adenovirus particle and wherein expression of the nucleic acid encoding the tissue kallikrein and the nucleic acid encoding the atrial natriuretic peptide results in production of tissue kallikrein and atrial natriuretic peptide. Thus, the present invention also provides an adenovirus comprising a nucleic acid comprising an isolated nucleic acid encoding tissue kallikrein and an isolated nucleic acid encoding atrial natriuretic peptide.

The present invention further contemplates a cell which has been genetically engineered to contain an exogenous nucleic acid encoding tissue kallikrein and an exogenous nucleic acid encoding atrial natriuretic peptide. Such cells can also be provided in a pharmaceutically acceptable carrier, as described herein.

Furthermore, the present invention provides a method for delivering tissue kallikrein and atrial natriuretic peptide to a cell comprising administering to the cell a nucleic acid encoding tissue kallikrein and atrial natriuretic peptide under conditions whereby the nucleic acid is expressed, thereby delivering tissue kallikrein and atrial natriuretic peptide to the cell. The nucleic acid can be delivered as naked DNA or in a vector (which can be a viral vector) or other delivery vehicles and can be delivered to the subject's cells *in vivo* and/or *ex vivo* by a variety of mechanisms well known in the art (e.g., uptake of naked DNA, viral infection, liposome fusion, endocytosis and the like). The cell can be any cell which can take up and express exogenous DNA,

including, but not limited to, a heart cell, kidney cell, liver cell, lung cell, adrenal gland cell, endothelial cell, neuronal cell, myoblast and hematopoietic stem cell.

Additionally provided is a method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension-associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the nonhypertension-associated renal disorder.

A method is also provided herein for treating a hypertension-associated renal disorder in a subject having a hypertension associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the hypertension-associated renal disorder.

The cell in the subject in which the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed to treat a nonhypertension-associated or hypertension-associated renal disorder can be any cell which can take up and express exogenous DNA, including, but not limited to, a heart cell, kidney cell, liver cell, lung cell, adrenal gland cell, endothelial cell, neuronal cell, myoblast and hematopoietic stem cell and is most preferably a kidney cell. In addition, the renal disorder to be treated by administration of a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide can be, but is not limited to, renal injury, nephrotoxicity, nonhypertension-associated or hypertension-associated renal damage, salt-induced renal damage, glomerulosclerotic lesions, tubular injury, drug-induced renal damage, chronic renal failure, acute renal failure, nephrotic syndrome and diabetic nephropathy.

Further provided is a method of treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the nonhypertension-associated cardiac disorder.

A method is also provided for treating a hypertension-associated cardiac disorder in a subject having a hypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the hypertension-associated cardiac disorder.

The cell in the subject in which the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed to treat a nonhypertension-associated or hypertension-associated cardiac disorder can be any cell which can take up and express exogenous DNA, including, but not limited to, a heart cell, kidney cell, liver cell, lung cell, adrenal gland cell, endothelial cell, neuronal cell, myoblast and hematopoietic stem cell and is most preferably a cardiac cell. In addition, the cardiac disorder to be treated by administration of a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide can be, but is not limited to, cardiac hypertrophy, nonhypertension-associated or hypertension-associated cardiac damage, heart failure after cardiac surgery, cardiac injury after myocardial infarction, myocardial ischemia, congestive heart failure, restenosis following angioplasty, cerebravascular disorders such as stroke and occlusive artery diseases and disorders.

The present invention provides a method for treating and/or preventing a cerebrovascular disorder (e.g., a stroke) in a subject having a cerebrovascular disorder and/or at risk of having a cerebrovascular disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein

and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing the cerebrovascular disorder.

Further provided is a method for treating and/or preventing an occlusive artery disorder (e.g., restenosis) in a subject having an occlusive artery disorder and/or at risk of having an occlusive artery disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing the occlusive artery disorder.

A method is also provided for inhibiting vascular smooth muscle cell growth and/or inhibiting neointimal formation in a blood vessel of a subject in need of inhibition of vascular smooth muscle cell growth and/or neointimal formation in a blood vessel, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby inhibiting vascular smooth muscle cell growth and/or neointimal formation in a blood vessel of the subject.

The present invention also provides a method for treating and/or preventing renal damage and/or renal injury caused by drug-induced and/or salt-induced nephrotoxicity in a subject having renal damage and/or renal injury caused by drug-induced and/or salt induced nephrotoxicity and/or at risk of having renal damage and/or renal injury caused by drug-induced and/or salt-induced nephrotoxicity, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing renal damage and/or renal injury caused by drug-induced and/or salt-induced nephrotoxicity.

Further provided is a method for stimulating renal tubular regeneration and/or reversing pre-existing renal injury in a subject in need of stimulation of renal tubular regeneration and/or reversal of pre-existing renal injury, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby stimulating renal tubular regeneration and/or reversing pre-existing renal injury in the subject. For example, the pre-existing renal injury can be caused by chronic renal failure, by drug-induced nephrotoxicity and/or by salt-induced nephrotoxicity.

Thus, the present invention also provides a method for treating and/or preventing chronic renal failure in a subject having chronic renal failure or at risk of having chronic renal failure, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing chronic renal failure in the subject.

The subject of the described methods of this invention can be any animal that utilizes tissue kallikrein and/or atrial natriuretic peptide and which can be treated for a nonhypertension-associated or hypertension-associated renal disorder and/or a nonhypertension-associated or hypertension-associated cardiac disorder. The animal can be a mammal and most preferably is a human.

The nucleic acid encoding TK and/or ANP can be administered to the cells of the subject either *in vivo* and/or *ex vivo*. If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols well known in the art. The nucleic acids of this invention can be introduced into the cells via any gene transfer mechanism, such as, for example, virus-mediated gene delivery, calcium phosphate mediated gene delivery, electroporation, microinjection or proteoliposomes. The transduced cells can then be infused (e.g., in a

pharmaceutically acceptable carrier) or homotopically transplanted back into the subject per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a subject.

For *in vivo* methods, the nucleic acid encoding TK and/or ANP can be administered to the subject in a pharmaceutically acceptable carrier as further described below.

In the methods described above which include the administration and uptake of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), the nucleic acids of the present invention can be in the form of naked DNA or the nucleic acids can be in a vector for delivering the nucleic acids to the cells for expression of the TK and/or ANP protein. The vector can be a commercially available preparation, such as an adenovirus vector (Quantum Biotechnologies, Inc. (Laval, Quebec, Canada). Delivery of the nucleic acid or vector to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the nucleic acid or vector of this invention can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

As one example, vector delivery can be via a viral system, such as a retroviral vector system which can package a recombinant retroviral genome (see e.g.,31,32). The recombinant retrovirus can then be used to infect and thereby deliver to the infected cells nucleic acid encoding TK and/or ANP. The exact method of introducing the altered nucleic acid into mammalian cells is, of course, not limited to the use of retroviral vectors. Other techniques are widely available for this procedure including the use of adenoviral vectors (33), adeno-associated viral (AAV) vectors (34), lentiviral vectors (35), pseudotyped retroviral vectors (36). Physical transduction techniques can

also be used, such as liposome delivery and receptor-mediated and other endocytosis mechanisms (see, for example, 37). This invention can be used in conjunction with any of these or other commonly used gene transfer methods.

The Examples of the present invention describe an adenovirus comprising a recombinant nucleic acid encoding TK and/or ANP inserted within an adenoviral nucleic acid, such that the recombinant nucleic acid is packaged in an adenovirus particle and the nucleic acid encoding TK and/or ANP is expressed within the cells into which the recombinant DNA is taken up, resulting in the production of TK and/or ANP. Various adenoviruses may be used in the compounds and methods described herein. For example, and as described in the Example contained herein, a nucleic acid encoding TK and/or ANP is inserted within the genome of adenovirus type 5. Similarly, other types of adenovirus may be used such as type 1, type 2, etc. For an exemplary list of the adenoviruses known to be able to infect human cells and which therefore can be used in the present invention, see Fields, *et al.* (38). Furthermore, it is contemplated that a recombinant nucleic acid comprising an adenoviral nucleic acid from one type adenovirus can be packaged using capsid proteins from a different type adenovirus.

The adenovirus of the present invention is preferably rendered replication deficient, depending upon the specific application of the compounds and methods described herein. Methods of rendering an adenovirus replication deficient are well known in the art. For example, mutations such as point mutations, deletions, insertions and combinations thereof, can be directed toward a specific adenoviral gene or genes, such as the E1 gene. For a specific example of the generation of a replication deficient adenovirus for use in gene therapy, see WO 94/28938 (Adenovirus Vectors for Gene Therapy Sponsorship) which is incorporated herein.

In the present invention, the TK and/or ANP gene can be inserted within an adenoviral genome and the TK and/or ANP encoding sequence can be positioned such that an adenovirus promoter is operatively linked to the TK and/or ANP insert such that the adenoviral promoter can then direct transcription of the TK and/or ANP nucleic acid, or the TK and/or ANP insert may contain its own adenoviral promoter. Similarly,

the TK and/or ANP insert may be positioned such that the nucleic acid encoding TK and/or ANP may use other adenoviral regulatory regions or sites such as splice junctions and polyadenylation signals and/or sites. Alternatively, the nucleic acid encoding TK and/or ANP may contain a different enhancer/promoter (e.g., CMV or RSV-LTR enhancer/promoter sequences as described in the Examples provided herein) or other regulatory sequences, such as splice sites and polyadenylation sequences, such that the nucleic acid encoding TK and/or ANP may contain those sequences necessary for expression of TK and/or ANP and not partially or totally require these regulatory regions and/or sites of the adenovirus genome. These regulatory sites may also be derived from another source, such as a virus other than adenovirus. For example, as described in the Examples herein, a polyadenylation signal from SV40 or BGH may be used rather than an adenovirus, a human, or a murine polyadenylation signal. The TK and/or ANP nucleic acid insert may, alternatively, contain some sequences necessary for expression of TK and/or ANP and derive other sequences necessary for the expression of the TK and/or ANP from the adenovirus genome, or even from the host in which the recombinant adenovirus is introduced.

As another example, for administration of TK and /or ANP genes to an individual in an AAV vector, the AAV particle can be directly injected intravenously. The AAV has a broad host range, so the vector can be used to transduce any of several cell types, but preferably cells in those organs that are well perfused with blood vessels. To more specifically administer the vector, the AAV particle can be directly injected into the target organ, such as muscle, liver or kidney. Furthermore, the vector can be administered intraarterially, directly into a body cavity, such as intraperitoneally, or directly into the central nervous system (CNS).

An AAV vector can also be administered in gene therapy procedures in various other formulations in which the vector plasmid is administered after incorporation into other delivery systems such as liposomes or systems designed to target cells by receptor-mediated or other endocytosis procedures. The AAV vector can also be incorporated into an adenovirus, retrovirus or other virus which can be used as the delivery vehicle.

As described above, the nucleic acid or vector of the present invention can be administered *in vivo* in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art.

The mode of administration of the nucleic acid or vector of the present invention can vary predictably according to the disease being treated and the tissue being targeted. For example, for administration of the nucleic acid or vector in a liposome, catheterization of an artery upstream from the target organ is a preferred mode of delivery, because it avoids significant clearance of the liposome by the lung and liver.

The nucleic acid or vector may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, topically or the like, although intravenous administration is typically preferred. The exact amount of the nucleic acid or vector required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity of the disease being treated, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every nucleic acid or vector. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein (see, e.g., Remington's Pharmaceutical Sciences; ref 39).

As one example, if the nucleic acid of this invention is delivered to the cells of a subject in an adenovirus vector, the dosage for administration of adenovirus to humans can range from about 10^7 to 10^9 plaque forming unit (pfu) per injection but can be as high as 10^{12} pfu per injection (59,60). Ideally, a subject will receive a single injection.

If additional injections are necessary, they can be repeated at six month intervals for an indefinite period and/or until the efficacy of the treatment has been established.

Parenteral administration of the nucleic acid or vector of the present invention, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution of suspension in liquid prior to injection, or as emulsions. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein.

In the methods of the present invention which describe the treatment of a nonhypertension-associated or hypertension-associated renal disorder, the efficacy of the treatment can be monitored according to clinical protocols well known in the art for monitoring the treatment of renal disorders. For example, such clinical parameters as renal sodium excretion, urine volume, urinary sediment and urine creatinine can be monitored according to methods standard in the art. Ideally, these parameters would be measured at about ten days after gene delivery. A clinician would look for reduction or absence of proteinuria, edema, hematuria, azotemia and casts as well as increased urine volume and sodium excretion as indicators of the efficacy of the treatment methods of this invention.

Furthermore, in determining the efficacy of treatment of a nonhypertension-associated or hypertension-associated cardiac disorder, a clinician would look for improvement in cardiac morphology and function as determined by electrocardiography, magnetic resonance imaging and/or positron emission tomography, the technologies of which are well known in the art. Ideally, these parameters would be measured at about 20 days after gene delivery.

In addition, the efficacy of treatment of restenosis according the methods provided herein can be monitored by angiocardiography, preferably at about 20 days after gene delivery.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

I. Adenovirus delivery of TK genes

Materials

Dahl salt-sensitive rats (Dahl-SS, male, 4 weeks old) (Sprague-Dawley Harlan, Indianapolis, IN) were used in this study. Rats were divided into three groups. The control group was fed a standard rat chow (0.4% NaCl)) (Harlan Teklad, Madison, WI). The experimental groups were fed a high salt diet (4% NaCl) (Harlan Teklad, Madison, WI). All rats had free access to water. Throughout the study period, all animals were housed in a room that was kept at constant temperature (25±1°C) and humidity (60±5%) and was lighted automatically from 8:00 am to 8:00 pm. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD).

Preparation of Replication-Deficient Adenovirus Vector Ad.CMV-cHK

Plasmid CMV-cHK was constructed as previous described (40) in which the expression of human tissue kallikrein cDNA was under the control of the cytomegalovirus (CMV) enhancer/promoter and was followed by a bovine growth hormone (BGH) polyA signal sequence. The transcription unit of CMV-cHK-polyA, including the CMV enhancer/promoter, the human tissue kallikrein cDNA and a BGH poly A signal sequence, was released from the CMV-cHK plasmid with Nae I /Nru I digestions. Plasmid pAd.CMV-cHK was constructed by inserting the released fragment into the adenovirus shuttle vector pAdLink.1 at an EcoRV site. The pAd.CMV-cHK plasmid DNA was purified using a Qiagen plasmid DNA kit. (Qiagen, Chatsworth, CA) and the purified DNA was sent to the Institute for Human Gene Therapy, Wistar Institute, Philadelphia for generation of adenovirus Ad.CMV-cHK harboring the CMV-cHK-polyA transcription unit. Adenovirus harboring the LacZ

gene under the control of the CMV enhancer/promoter (Ad.CMV-LacZ) was purchased from the Institute for Human Gene Therapy.

Intravenous Delivery of Adenoviral Vectors Ad.CMV-cHK and Ad.CMV-LacZ

Seven Dahl-SS rats of each group fed with a high salt diet containing 4% NaCl were intravenously (IV) injected with either Ad.CMV-cHK or Ad.CMV-LacZ at a dosage of 1.2×10^{10} pfu (plaque formation unit) per rat through the tail vein. During the experimental period, blood was collected daily from the tail vein for the first eight days and every two days from 9 to 36 days after injection. Rat serum samples were frozen at -80°C until the expression level of human tissue kallikrein could be examined by ELISA.

Blood Pressure Measurement

The systolic blood pressure of rats was measured with a manometer-tachometer (model KN-210-1; Narco Bio-systems) using the tail-cuff method (41). Unanesthetized rats were introduced into a plastic holder mounted on a thermostatically controlled warm plate, which was maintained at 33-35°C during measurement. An average of ten readings was taken for each animal after they became acclimated to the environment.

Urine Collection and Analysis of Physiological Parameters

Twenty-four hour urine of rats was collected in metabolic cages at 9, 17, and 23 days post gene delivery. Rats were fed a 4% NaCl diet for three hours before being placed in metabolic cages supplied with drinking bottles. To eliminate contamination of urine samples, animals received only water during the 24 hour collection period. Urine was collected and centrifuged in a microfuge at 1,000x g to remove particles. The volume of the supernatant was measured and stored at -20°C until analysis. Each sample was used to measure urinary kinin, cyclic GMP (cGMP), sodium and potassium output, urinary creatinine and total protein.

Tissue Preparation

At the end of the experiment, all rats were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight. Blood samples were collected by

direct cardiac puncture and chilled at 4°C overnight. The blood samples were centrifuged at 1,000 x g for 20 min and sera were removed and frozen at -20°C. At the same time, rats were perfused with normal saline (0.9% NaCl) via the heart. The whole heart, left ventricle and left and right kidneys were removed, blotted and weighed. Tissues of interest were removed and total RNAs were extracted by the guanidine isothiocyanate-cesium chloride ultracentrifugation method. The extracted RNA was quantified spectrophotometrically by absorbance at 260 nm, dissolved in diethyl pyrocarbonate-treated water and stored at -80°C for further use.

RT-PCR Southern Blot Analysis of Human Tissue Kallikrein mRNA

The reaction mixture for reverse transcription (RT) contained 1 μg of total RNA from Dahl-SS rats, 10 pmols of 3' primer (5'-CTTCACATAAGACAGCA -3' of the human tissue kallikrein gene) (SEQ ID NO:1), 16 nmols of dNTP, 0.2 μmole of DTT, 4 μl of 5x reverse transcription buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 15 mmol/L $MgCl_2$) and 200 U of Maloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD), in a total volume of 20 μl. The RT reaction mixture was incubated at 37°C for 1 hour to synthesize the first strand of cDNA. Ten pmol of 5'-primer (5'-AACACAGCCCAGTTTGT-3') (SEQ ID NO:2), 5 μl of 10x PCR buffer and 0.5 U of Taq DNA polymerase were added to the RT mixture to a total volume of 50 μl followed by 30 cycles of polymerase chain reaction (PCR) (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) with mineral oil. Ten μg of PCR products were subjected to a Southern blot analysis. A specific oligonucleotide (5'-GACCTCAAAATCCTGCC-3') (SEQ ID NO:3) was used as an internal probe for hybridization at 42°C. Hybridization was carried out in a solution containing 6x SSC (900 mmol/L NaCl, 9 mmol/L sodium citrate, pH 7.0), 5x Denhardt's solution, 0.5% SDS, 100 μg/ml herring sperm DNA and ^{32}P-labeled oligonucleotide primer. The blot was washed to a final stringency of 1x SSC at 42°C. The blot was exposed to Kodak X-Omat film at -80°C (Eastman Kodak Co., Rochester, NY).

Enzyme-linked Immunosorbent Assay (ELISA) for Human Tissue Kallikrein

Tissues were immersed in PBS (phosphate-buffered saline; pH 7.0) and homogenized with a Polytron (Brinkmann Instruments). The levels of immunoreactive

human tissue kallikrein in each tissue extract, rat urine and rat sera were determined by an ELISA specific for human tissue kallikrein. Anti-human tissue kallikrein IgG was coupled to biotin. Microtiter plates (96 well) were coated with anti-kallikrein IgG (2 μg/ml, 100 μl/well) at 4°C overnight. Purified human tissue kallikrein standard (0.04-2.5 ng) and rat tissue extracts were added to individual wells in a total volume of 100 μl of PBS containing 0.05% Tween-20 and 0.5% gelatin. Biotin-labeled anti-human tissue kallikrein IgG was added in each well at a concentration of 1 μg/ml in a total of 100 μl. Peroxidase-avidin (1 μg/ml in a total of 100 μl) was added and incubated at 37°C for 60 min. The color reaction was performed by adding 100 μl/well of freshly prepared substrate solution [0.03% 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.03% H_2O_2 in 0.1 mol/L citrate buffer, pH 4.3] and incubating at room temperature for 30 min. The plates were read at 404 nm with a Titertek plate reader (53).

Radioimmunoassay (RIA) of Urinary Kinin

Urinary kinins levels were determined by a direct radioimmunoassay as described (58). Briefly, 100 μl of ^{125}I-labeled bradykinin (10,000 cpm/100 μl), 100 μl rabbit antiserum against bradykinin (at a 1:100,000 dilution), 100 μl diluted sample and 100 μl 0.1% egg albumin assay buffer (0.1% egg albumin, 10 mM EDTA, 10 mM 1,10-phenanthroline in PBS, pH 7.0) in a final volume of 400 μl were incubated at 4°C overnight. After addition of 400 μl 1% bovine-γ-globulin and 800 μl 25% polyethylene glycol in PBS to the reaction mixture, free and antibody-bound bradykinin were separated by centrifugation at 5000 rpm for 30 min. The standard bradykinin used ranged from 4.0 pg to 0.5 ng.

Radioimmunoassay for cGMP

The procedure for assay of cGMP was conducted according to the general procedure of Harper and Brooker (43,44) as modified by Gettys et al. (45,46). The iodination was performed by adding 20 μl of 50 mmol/L phosphate buffer to 25 μg (in 10 μl of 0.5 mol/L potassium phosphate buffer, pH 7.4) of 2'-O-monosuccinylguanosine 3':5'-cyclic monophosphate tyrosyl methyl ester (cGMP-TME, Sigma Chemical Co.), followed by 5 μl of Na^{125}I (0.5 mCi). Twenty μl

of 0.01% chloramine T (Sigma Chemical Co.) solution was added to the mixture and incubated for 30 sec. The reaction was stopped by adding 50 µl of 25% acetic acid. The resultant mixture was subjected to C-18 reversed phase HPLC to separate the iodinated cGMP-TME from free iodine. Standards (10, 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0.08 nmol/L) and urine samples were acetylated by adding 20 µl of triethylamine and 10 µl of acetic anhydride to each tube. Fifty µl aliquots of each acetylated standard and sample, 25 µl of diluted cGMP antiserum (1:14,400) and 25 µl of iodinated cGMP (15,000 cpm) were mixed in the assay tubes and incubated overnight at 4°C. The assay was stopped by adding 50 µl of 5x diluted human plasma containing 4 mmol/L EDTA, followed by 1 ml of cold 12% polyethylene glycol. The tubes were vortexed and incubated at 4°C for 1 hour before spinning for 20 min at 1,000x g at 4°C. The supernatant was aspirated and another 1 ml of 12% polyethylene glycol was added gently to each tube. Tubes were centrifuged as before, the supernatants were aspirated and the tubes were counted in a Gamma counter.

Morphological and Histological Investigation

Rats were anesthetized with pentobarbital (50 mg/kg body weight) and hearts and kidneys were removed, cleaned, washed in saline, blotted and weighed. Sections of the kidney and heart were preserved in 10% buffered formaldehyde solution and paraffin embedded. Five µm-thick sections were cut and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) and analyzed microscopically and morphometrically. For linear measurements (diameters), an ocular micrometer was calibrated against a stage micrometer and conversion factors were calculated for low (4x objective) and high (45x objective) magnifications. All the sections were evaluated by independent personnel without the prior knowledge of the group in which the rats belonged.

Statistical Analysis

Data were analyzed using standard statistical methods. Repeated blood pressure measurements were taken after gene delivery for comparison between control and experimental groups at each time point with the use of unpaired Student's t-test and

ANOVA and Fisher's protected least significant differences. Group data are expressed as mean±SEM. Values were considered significantly different at a value of $P<0.05$.

Blood Pressure Reduction after Intravenous Injection of Ad.CMV-cHK Adenovirus

Dahl-SS rats (4 weeks old) were fed a high salt (4% NaCl) diet or normal rat chow (0.4% NaCl) as controls for 1-2 weeks until blood pressure differences between these two groups reached over 20 mmHg. Rats on a high salt diet were then divided into two groups and injected with either Ad.CMV-cHK or Ad.CMV-LacZ adenovirus through the tail vein. Systolic blood pressures of these rats were monitored every other day after injection for one week and then once a week. Delivery of the human tissue kallikrein gene caused a significant reduction of blood pressure two days after injection. A maximal blood pressure reduction in rats injected with Ad.CMV-cHK was observed 17 days after gene delivery as compared to that of control rats injected with Ad.CMV-LacZ (174.8±0.4 vs. 189.0±0.9 mmHg, mean±SEM, n=6, $P<0.01$). The difference in the blood pressure between the control group and the group receiving kallikrein gene delivery persisted until four weeks post injection.

Expression of Human Tissue Kallikrein after Gene Delivery.

Expression of the human tissue kallikrein mRNA in Dahl-SS rats after gene delivery was detected by RT-PCR followed by Southern blot analysis using three oligonucleotides specific for human tissue kallikrein. Total RNAs were prepared from tissues of rats 12 days after gene delivery. The liver and kidney expressed the most human kallikrein mRNA followed by adrenal gland, heart and lung. The RT-PCR products from rats receiving the Ad.CMV-LacZ gene did not hybridize to the human tissue kallikrein gene probe. Similar levels of β-actin mRNA were detected in tissues of both experimental and control groups, indicating the quality of these RNA samples. These results indicate that Southern blot analysis is specific for human tissue kallikrein and that endogenous rat tissue kallikrein family members do not interfere with the assay.

Expression levels of human tissue kallikrein in Dahl-SS rats were measured by ELISA. Immunoreactive human tissue kallikrein was detected in the kidney

(242.8±39.4 pg/mg protein, n=7) and liver (147.8±21.7 pg/mg protein, n=7) 31 days after kallikrein gene delivery. Linear displacement curves for immunoreactive kallikrein in the liver and kidney were parallel with the standard curve of human tissue kallikrein, indicating their immunological identity. Human tissue kallikrein was not detected by ELISA in the liver or kidney of rats receiving control adenovirus, Ad.CMV-LacZ. The results showed that rabbit anti-human tissue kallikrein antibody is specific for human kallikrein and has no cross-reaction with rat tissue kallikrein gene family members.

Time-Dependent Expression of Human Tissue Kallikrein

Following intravenous injection of Ad.CMV-cHK adenovirus, human tissue kallikrein levels in rat sera and urine, collected at different time periods, were measured by ELISA. The highest level of immunoreactive human tissue kallikrein in rat serum was 254±0.1 ng/ml on the third day after gene delivery. Human tissue kallikrein expression lasted for 5-6 weeks and decreased gradually from day 4 to day 36 post injection. Also, immunoreactive human tissue kallikrein was detected in the urine of Dahl-SS rats and decreased gradually after gene delivery at day 9, 17 and day 21, respectively (5.8±1.3, 2.6±0.7 and 0.8±0.4 μg/100 g body weight per day). Linear displacement curves for immunoreactive kallikrein in rat sera and urine were parallel with the standard curve of human tissue kallikrein, indicating their immunological identity.

Effects of Ad.CMV-cHK Gene Delivery on Physiological Parameters

Table 1 shows the effects of Ad.CMV-cHK gene delivery on physiological parameters of Dahl-SS rats on day 9. Urine excretion was significantly increased in rats receiving Ad.CMV-cHK adenovirus as compared to rats injected with Ad.CMV-LacZ adenovirus (11.7±2.3 versus 3.5±0.9 ml/100 g body weight per day, mean±SEM; n=6, $P<0.05$). Similarly, water intake was significantly increased in rats receiving kallikrein gene delivery as compared to control rats (12.9±2.0 versus 6.8±0.9 ml/100 g body weight per day, mean±SEM; n=6, $P<0.05$). Urinary sodium output in the Ad.CMV-cHK group significantly increased as compared to the control Ad.CMV-LacZ group (0.40±0.03 versus 0.27±0.02 mmol/100 g body weight per day,

mean±SEM; n=6, P<0.05). Following kallikrein gene delivery, urinary kinin levels increased by 5-fold (50.7±6.7 vs. 9.9±2.2 ng/100 g body weight per day, mean±SEM; n=6, P<0.01) and urinary cGMP levels also increased significantly as compared to the Ad.CMV-LacZ group (10.7±2.1 vs. 7.6±1.0 nmol/100 g body weight per day, mean±SEM; n=6, P<0.05). However, no significant changes in heart rate, body weight, urinary potassium output, urinary creatinine and total protein excretion were observed between Ad.CMV-cHK and Ad.CMV-LacZ groups.

Morphological Changes in the Heart after Gene Delivery

Table 2 shows that the ratio of left ventricular weight to 100 g body weight is significantly decreased in rats injected with Ad.CMV-cHK as compared to control rats injected with Ad.CMV-LacZ (0.36±0.02 versus 0.38±0.04, mean±SEM; n=6, P<0.05). In addition, the diameter of cardiac myocytes in the group receiving kallikrein gene transfer is significantly reduced as compared to that of the Ad.CMV-LacZ group (16.8±0.3 versus 19.1±0.6 μm, mean±SEM; n=80, P<0.05). No significant changes in the ratio of whole heart weight, left renal mass, or right renal mass were detected (Table 2). These results indicate that kallikrein gene delivery attenuated salt-induced cardiac hypertrophy in Dahl-SS rats after salt loading.

Morphological Changes in the Kidney after Gene Delivery

Kallikrein gene delivery was shown to impart protective effects on salt-induced renal damage in Dahl-SS rats. The glomerular basement membrane thickening in rats receiving kallikrein gene transfer was less than in control rats receiving the LacZ gene. Damage to the proximal tubular brush border and dilatation of tubules in rats receiving kallikrein gene delivery was significantly reduced as compared to control rats. Major renal injury induced by high salt loading in the group injected with the control adenovirus, Ad.CMV-LacZ, was found in the dilated renal tubules, especially in proximal tubules with decreased cell height and increased tubular diameter. Dilated tubular lumens accumulated colloid or protein casts which were less evident in the group receiving kallikrein gene delivery. In addition, severe inflammatory cell infiltration around renal arterioles and in interstitial tissues was observed in the control

Ad.CMV-LacZ group. These results indicate that kallikrein gene delivery protected Dahl-SS rats from salt-induced renal injury.

The present study shows that a continuous supply of tissue kallikrein by somatic gene delivery into Dahl-SS rats fed a high salt diet resulted in a sustained reduction of blood pressure as well as attenuation of cardiac hypertrophy and renal injury. The expression of human tissue kallikrein was detected in tissues involved in cardiovascular and renal function, such as the kidney, heart, adrenal gland and lung. High levels of immunoreactive human tissue kallikrein were detected in the serum and urine of rats receiving gene delivery, indicating secretion of human kallikrein from the liver and kidney.

The study described herein further demonstrates that tissue kallikrein is expressed in the kidney and heart of rats receiving the gene in an adenovirus vector. Dahl-SS rats fed a high salt diet exhibit salt-induced renal damage, including glomerular basement membrane thickening, renal tubular dilation and disruption of the proximal tubular brush border. Kallikrein gene transfer exerts a protective effect against this damage. In addition, cardiac hypertrophy in Dahl-SS rats was also alleviated via tissue kallikrein gene delivery as evidenced by reduction of the diameter of cardiac myocytes and left ventricular weight.

II. Adenovirus delivery of atrial natriuretic peptide (ANP) gene

Materials

Dahl salt-sensitive rats (Dahl-SS, male, four weeks old) (Sprague-Dawley Harlan, Indianapolis, IN) were used in this study. Rats were divided into three groups. The control group was fed a standard rat chow (0.4% NaCl) (Harlan Teklad, Madison, WI). The experimental groups were fed a high salt diet (4.0% NaCl) (Harlan Teklad, Madison, WI). All rats had free access to water. Throughout the study period, all animals were housed in a room that was kept at constant temperature (25±1°C) and humidity (60±5%) and was lighted automatically from 8:00 AM to 8:00 PM. All procedures complied with the standards of care and use of animal subjects as stated in

the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD).

Preparation of Replication-deficient Adenovirus Vector Ad.RSV-cANP

Plasmid RSV-cANP was constructed as previous described (47), in which the expression of human atrial natriuretic peptide cDNA was under the control of the Rous sarcoma virus long terminal repeat (RSV-LTR) enhancer/promoter and was followed by a SV40 polyA signal sequence. The transcription unit of RSV-cANP-polyA, including the RSV-LTR enhancer/promoter, the human atrial natriuretic peptide cDNA and Simian virus 40 poly A signal sequence, was released from the RSV-cANP plasmid by Sal I digestion. Plasmid pAd.RSV-cANP was constructed by inserting the released fragment into the adenovirus shuttle vector pAdLink.1 at a Sal I site. The pAd.RSV.cANP plasmid DNA was purified using a Qiagen plasmid DNA kit. (Qiagen, Chatsworth, CA) and the purified DNA was sent to the Institute for Human Gene Therapy, Wistar Institute, Philadelphia for generation of adenovirus Ad.RSV.cANP harboring the RSV-cANP-polyA transcription unit. Adenovirus harboring the LacZ gene under the control of the cytomegalovirus (CMV) enhancer/promoter (Ad.CMV-LacZ) was purchased from the Institute for Human Gene Therapy.

Intravenous Delivery of Adenoviral Vectors Ad.RSV-cANP and Ad.CMV-LacZ

Seven Dahl-SS rats of each group fed with a high salt diet containing 4% NaCl were intravenously (IV) injected with either Ad.RSV-cANP or Ad.CMV-LacZ at a dosage of 1.0×10^{10} pfu (plaque formation unit) per rat through the tail vein. During the experimental period, blood was collected daily from the tail vein for the first 8 days and every 2 days from 9 to 36 days after injection. Rat serum samples were frozen at -80°C for examining the expression level of human ANP.

Blood Pressure Measurement

The systolic blood pressure of rats was measured with a manometer-tachometer (Nastume KN-210; Nastume Seisakusho Co. Ltd., Tokyo, Japan) using the tail-cuff method (41). Unanesthetized rats were introduced into a plastic holder mounted on a thermostatically controlled warm plate, which was maintained at 33-35°C during

measurement. An average of ten readings was taken for each animal after they became acclimated to the environment.

Urine Collection and Analysis of Physiological Parameters

Twenty-four hour urine of rats was collected in metabolic cages at 5, 11, and 25 days post gene delivery. Rats were fed a 4% NaCl diet for three hours before being placed in metabolic cages supplied with drinking bottles. To eliminate contamination of urine samples, animals received only water during the 24 hour collection period. Urine was collected and centrifuged in a microfuge at 1,000 x g to remove particles. The volume of the supernatant was measured and stored at -20°C until analysis. Each sample was used to measure urinary cyclic GMP (cGMP), sodium output, total protein and kinin.

Tissue Preparation

At the end of the experiment, all rats were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight. Blood samples were collected by direct cardiac puncture and chilled at 4°C overnight. The blood samples were centrifuged at 1,000 x g for 20 min and sera were removed and frozen at -20°C. At the same time, rats were perfused with normal saline (0.9% NaCl) via the heart. The whole heart, left ventricle and left and right kidneys were removed and weighed. Tissues of interest were removed and total RNAs were extracted by the guanidine isothiocyanate-cesium chloride ultracentrifugation method. The extracted RNA was quantified spectrophotometrically by absorbance at 260 nm, dissolved in diethyl pyrocarbonate-treated water and stored at -80°C for further use.

Radioimmunoassay (RIA) for Human ANP

The level of human atrial natriuretic peptide in each tissue extract was determined by a RIA specific for human ANP. Ten micrograms of human synthetic ANP (Ser 99-Tyr 126) (Sigma Chemical Co., St. Louis, MO) was labeled with 1 mCi of ^{125}-Iodine which was iodinated with iodogen for 10 min at room temperature. The iodinated ANP in 250 mmol/L sodium phosphate buffer, pH 7.0, was separated on a reverse-phase C-18 HPLC column in an acetonitrile gradient. ^{125}I-ANP labeled tracer

which was eluted from the column at 19-20 min post-injection was identified by antibody titration. Serial dilutions of standard ANP (10 pg-1280 pg) or tissue extracts (100 µl) were incubated with goat-anti-human ANP antiserum (1: 1500 dilution, Sigma Chemical Co., St. Louis, MO) in a solution containing 0.01 mol/L PBS, pH 7.4, 0.3% BSA, 0.1% Triton X100, 0.1 mmol/L EDTA and 0.1% sodium azide and ^{125}I-ANP tracer (10,000 cpm in 100 µl) in a total volume of 400 µl for 18-24 hours at 4°C. The reaction was stopped by adding 800 ml of 25% polyethylene glycol (PEG) in PBS containing 0.1% sodium azide and 400 ml of 1% bovine gamma-globulin in PBS containing 0.1% sodium azide. The radioactivity of the precipitate was determined in a gamma counter.

Radioimmunoassay (RIA) for cyclic GMP

The procedure for assay of cGMP was conducted as previously described (48). The iodination was performed by adding 20 µl of 50 mmol/L phosphate buffer to 25 µg (in 10 µl of 0.5 mol/L potassium phosphate buffer, pH 7.4) of 2'-O-monosuccinylguanosine 3':5'-cyclic monophosphate tyrosyl methyl ester (cGMP-TME, Sigma Chemical Co.), followed by 5 µl of Na^{125}I (0.5 mCi). Twenty µl of 0.01% chloramine T (Sigma Chemical Co.) solution was added to the mixture and incubated for 30 sec. The reaction was stopped by adding 50 µl of 25% acetic acid. The resultant mixture was subjected to C-18 reversed phase HPLC to separate the iodinated cGMP-TME from free iodine. Standards (10, 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0.08 nmol/L) and urine samples were acetylated by adding 20 µl of triethylamine and 10 µl of acetic anhydride to each tube. Fifty µl aliquots of each acetylated standard and sample, 25 µl of diluted cGMP antiserum (1:14,400) and 25 µl of iodinated cGMP (15,000 cpm) were mixed in the assay tubes and incubated overnight (16 hours) at 4°C. The assay was stopped by adding 50 µl of 5x diluted human plasma containing 4 mmol/L EDTA, followed by 1 ml of cold 12% polyethylene glycol. The tubes were vortexed and incubated at 4°C for 1 hour before spinning for 20 min at 1,000x g at 4°C. The supernatant was aspirated and another 1 ml of 12% polyethylene glycol was added gently to each tube. Tubes were centrifuged as before, the supernatants were aspirated and the tubes were counted in a Gamma counter.

Measurements of Renal Function: Glomerular Filtration Rate and Renal Blood Flow

At the end of the experiment, rats were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight and placed on a heated table for maintenance of body temperature at 37°C (49). After tracheotomy, a cannula was placed in a jugular vein for infusion of liquids. A cannula was placed in the left femoral artery for measurement of blood pressure and for blood sampling. The bladder was cannulated to allow urine collection from both kidneys. During the experiment, 0.9% NaCl containing inulin and 2% PAH was infused through the jugular vein at a constant infusion rate of 1.2 ml/hour. Forty-five minutes was allowed for the preparation to reach a steady state before study. Timed urine collections were obtained, with blood collected (0.7 ml) between pairs of clearance periods.

At the end of each experiment, the kidneys were excised, blotted and weighed. Urine volume was determined gravimetrically. Inulin and PAH concentrations were determined by modified anthrone and colorimetric methods, respectively (50,51). GFR was determined from the clearance of inulin. RBF was determined from the clearance of PAH. Clearance data were normalized to kidney weight.

Morphological and Histological Investigation

Rats were anesthetized with pentobarbital (50 mg/kg body weight) and hearts, aortas and kidneys were removed, cleaned, washed in saline and weighed. Sections of the kidney, heart and aorta were preserved in 10% buffered formaldehyde solution and paraffin embedded. Five μm-thick sections were cut and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) and analyzed microscopically and morphometrically. For linear measurements (diameters), an ocular micrometer was calibrated against a stage micrometer and conversion factors were calculated for low (4x objective) and high (45x objective) magnifications. All the sections were evaluated by independent personnel without the prior knowledge of the group in which the rats belonged.

Statistical Analysis

Data were analyzed using standard statistical methods. Repeated blood pressure measurements were taken after gene delivery for comparison between control and experimental groups at each time point using the unpaired Student's *t*-test and ANOVA and Fisher's protected least significant differences (PLSD). Group data are expressed as mean±SEM. Values were considered significantly different at $P<0.05$.

Blood Pressure Reduction after Intravenous Injection of Ad.RSV-cANP Adenovirus

Dahl-SS rats (4 weeks old) were fed a high salt (4% NaCl) diet or normal rat chow (0.4% NaCl) as controls for 1-2 weeks until blood pressure differences between these two groups reached over 20 mmHg. Dahl-SS rats on a high salt diet were then divided into two groups and intravenously injected with either Ad.RSV-cANP or Ad.CMV-LacZ through the tail vein. Blood pressures of these rats were monitored every 2-3 days after injection for six weeks. Delivery of the human ANP gene caused a significant reduction of blood pressure three days after injection and the effect lasted for five and one half weeks, as compared to the rats receiving Ad.CMV.LacZ adenovirus. A maximal blood pressure reduction in Dahl-SS rats injected with Ad.RSV.cANP was observed 27 days after gene delivery as compared to that of control rats injected with Ad.CMV-lacZ (178.7±5.1 vs. 211.5±5.9 mmHg, mean±SEM, n=6, $P<0.01$). However, the blood pressure of control rats with a normal salt diet remained around 135-145 mmHg throughout the experimental period.

Expression of Human Atrial Natriuretic Peptide after Gene Delivery.

Expression levels of human ANP in Dahl-SS rats were analyzed by a radioimmunoassay specific for human ANP. Immunoreactive human ANP was detected in the heart (53.4 ng/mg protein), lung (2.4 ng/mg protein) and kidney (13.04 ng/mg protein) seven days after intravenous injection of the human ANP gene. Linear displacement curves for immunoreactive ANP in the heart, lung and kidney of Dahl-SS rats were parallel with the standard curve of human ANP, indicating their immunological identity. A serial dilution of heart, lung and kidney extracts from control rats injected with Ad.CMV.LacZ did not show parallelism with the human ANP standard. These results indicate that goat anti-human ANP antibody has some cross-

reactivity with rat ANP, however, human and rat ANP are not immunologically identical and are distinguished in the RIA.

Effects of Ad.RSV-cANP Gene Delivery on Physiological Parameters

Table 3 shows the effects of Ad.RSV-cANP gene delivery on physiological parameters of Dahl-SS rats on day 11. Urine excretion was significantly increased in the group receiving Ad.RSV-cANP adenovirus as compared to the group injected with Ad.CMV-LacZ adenovirus (16.7±2.7 versus 11.8±1.7 ml/100 g body weight per day, mean±SEM; n=6, $P<0.05$). However, water intake was slightly but not significantly increased in the group receiving Ad.RSV-cANP as compared to the control group (12.0±3.0 versus 9.9±2.7 ml/100 g body weight per day, mean±SEM; n=6). Also, urinary sodium output in the Ad.RSV-cANP group significantly increased by 64% as compared to those rats receiving Ad.CMV-LacZ (0.72±0.06 versus 0.44±0.07 mmol/100 g body weight per day, mean±SEM; n=6, $P<0.05$). Urinary cGMP levels increased by 4.8 fold as compared to the Ad.CMV.LacZ group (36.43±13.73 versus 7.62±1.02 nmol/100 g body weight per day, mean±SEM, n=6, $P<0.05$). However, no significant changes in heart rate, body weight, water intake and urinary total protein excretion were observed between Ad.RSV-cANP and Ad.CMV-LacZ groups 11 days after gene delivery.

Morphological Changes in the Heart and Kidney after Atrial Natriuretic Peptide Gene Delivery

Table 4 shows that the ratio of whole cardiac weight to 100 g body weight was significantly decreased in rats injected with Ad.RSV.cANP as compared to control rats injected with Ad.CMV-LacZ (0.43±0.03 versus 0.54±0.04, mean±SEM; n=6, $P<0.05$). Also, the ratio of left ventricular weight to 100 g body weight was significantly decreased in rats injected with Ad.RSV.cANP as compared to control rats injected with Ad.CMV.LacZ (0.32±0.003 versus 0.41±0.04, mean±SEM; n=6, $P<0.05$). In addition, the diameter of cardiomyocytes in the group receiving ANP gene delivery was significantly reduced as compared to that of the Ad.CMV-LacZ group (15.22±0.13 versus 19.07±0.16 μm, mean±SEM; n=120, $P<0.05$). Similarly, the ratio of average renal mass to 100 g body weight was also significantly decreased in rats injected with

Ad.RSV.cANP as compared to that of control rats injected with Ad.CMV.LacZ (0.55±0.03 versus 0.61±0.01, mean±SEM; n=6, P<0.05). However, a normal salt diet did not cause severe cardiac or left ventricular hypertrophy and enlargement of average renal mass and the size of cardiomyocytes in control rats with 0.4% NaCl diet as compared to those in high salt loading groups with either Ad.RSV-cANP or Ad.CMZ-LacZ injection (Table 4). No significant differences in the aortic weight to 100 g body weight were observed among these three groups. These results indicate that salt-induced cardiac hypertrophy and enlargement of renal mass in Dahl-SS rats were attenuated by ANP gene delivery.

Effects on Renal Function after Atrial Natriuretic Peptide Gene Delivery

After human ANP gene delivery, glomerular filtration rate in Ad.RSV.cANP-injected rats significantly increased by 3.3 fold as compared to rats receiving Ad.CMV-LacZ injection (0.56±0.06 versus 0.17±0.05 ml/min/g kidney weight, mean±SEM, n=3 or 4, P<0.05) and significantly decreased by 2 fold as compared to control rats with a normal salt diet (0.56±0.06 versus 1.19±0.17 ml/min/g kidney weight, mean±SEM, n=3 or 4, P<0.05) (Table 5). Also renal blood flow in Ad.RSV-cANP-injected rats was significantly increased by 38 fold as compared to rats receiving Ad.CMV-LacZ injection (6.95±0.31 versus 0.18±0.07 ml/min/g kidney weight, mean±SEM, n=3 or 4, P<0.05), but no significant change was observed between ANP and control groups. The PAH clearance through both kidneys of Ad.RSV-cANP-injected rats, though with a high salt loading, remained to be as good as that of control rats, but it significantly decreased by up to 46 fold in Ad.CMV-LacZ injected rats as compared to the Ad.RSV-cANP group (0.04±0.02 versus 1.78±0.71 mg/min/g kidney weight, mean±SEM, n=3 or 4, P<0.05). However, urine flow rate was slightly but not significantly decreased in the Ad.RSV-cANP group as compared to the Ad.CMV-LacZ group and control group with a normal salt diet (2.5±0.65 versus 5.61±1.77 and 6.12±2.75 µl/min/g kidney weight, mean±SEM, n=3 or 4). In addition, hematocrit in the Ad.CMV-LacZ group was also decreased by 22% as compared to Ad.RSV-cANP and control groups (44±6 versus 56±2 and 57±3%, mean±SEM, n=3 or 4, P<0.05). These results indicate that salt-induced renal damage causing the loss of renal functions including glomerular filtration and tubular reabsorption of water and electrolytes were attenuated after human

ANP gene delivery. The decreased hematocrit in the Ad.CMV-LacZ group with high salt loading was due mainly to total fluid volume expansion and sodium retention.

Histological Changes in the Kidney and Heart after Atrial Natriuretic Peptide Gene Delivery

ANP gene deliver imparts a protective effect on salt-induced renal damage in Dahl-SS rats. In the Ad.CMV-LacZ group, the major cortical damage included thickening of glomerular basement, especially obvious in PAS-stained tissue sections. Medullary injury induced by high salt loading was apparent in accumulated colloid or protein casts of dilated renal tubules. In addition, arterial thickening with proliferation in all layers was found in the LacZ group. Abnormal interstitial and epithelial cells were seen in the salt loaded LacZ group. These lesions, which undoubtedly cause renal dysfunction, were rarely seen in the Ad.RSV-cANP-treated rats. These results indicate that adenovirus-mediated gene delivery of ANP protected Dahl-SS rats fed a high salt diet from salt-induced renal injury and dysfunction.

A protective effect of ANP gene delivery on salt-induced left ventricular hypertrophy in Dahl-SS rats was also observed. The high salt loading, which caused sodium retention and hypertension, resulted in an enlarged average diameter of cardiomyocytes in Dahl salt sensitive rats with Ad.CMV-LacZ injection. Diffused interstitial proliferation was found in the LacZ group but not in the group that received ANP gene delivery.

These data demonstrate that expression of ANP was detected in tissues relevant to cardiovascular and renal function, such as the kidney, lung and heart. Furthermore, the present study shows that a continuous supply of ANP by adenovirus-mediated gene delivery into Dahl-SS rats fed a high salt diet resulted in a sustained reduction of blood pressure as well as attenuation of cardiac hypertrophy and renal injury. ANP gene transfer in Dahl-SS rats fed a high salt diet exerts protective effects in salt-induced renal damage including thickening in the basement membrane of glomeruli, dilated renal tubules with the accumulation of colloid or protein casts, preservation of the brush border of the proximal tubules.

III. Delivery of TK gene in adenovirus vector to treat nephrotoxicity

Preparation of replication-deficient adenoviral vector Ad.CMV-cHK

Plasmid CMV-cHK was constructed as previously described (52), in which the expression of human tissue kallikrein cDNA was under the control of the cytomegalovirus enhancer/promotor and was followed by the bovine growth hormone gene polyadenylation signal sequence. Plasmid pAd.CMV-cHK was constructed by inserting the CMV-cHK-pA transcription unit into the adenoviral shuttle vector pAdLink.1 at an EcoRV site. The pAd.CMV-cHK plasmid DNA was purified using a Qiagen plasmid DNA kit (Qiagen, Chatsworth, CA) and the purified DNA was sent to the Institute for Human Gene Therapy, Wistar Institute, Philadelphia, PA for generating adenovirus Ad.CMV-cHK harboring the CMV-cHK-polyA transcription unit. Adenovirus harboring the LacZ gene under the control of the cytomegalovirus enhancer/promoter (Ad.CMV-LacZ) was obtained from the Institute for Human Gene Therapy.

Animal treatment

Sprague-Dawley rats (male, 200-220 g body weight, Harlan Sprague Dawley, Inc., Indianapolis, IN) were used in this experiment. The rats were housed at a constant room temperature with a 12 hr light/dark cycle and had free access to tap water and rat chow. The rats were divided at random into four groups with six animals in each group. The first group was injected subcutaneously with normal saline (0.9% NaCl). The other three groups received gentamycin sulfate (Sigma Chemical, St. Louis, MO) subcutaneously at a dose of 80 mg/kg in saline solution. The injection was carried out for 10 consecutive days. The third group was injected with adenovirus Ad.CMV-LacZ and the fourth group with adenovirus Ad.CMV-cHK (4×10^{10} plaque-forming units) *via* the tail vein on the first day of gentamycin treatment.

Blood pressure measurement

Systolic blood pressure was measured with a photoelectric tail cuff device (Natsume Co., Tokyo, Japan). This device requires minimal warming of the rats (usually <20 min) prior to blood pressure determination and a brief period of restraint

in a plastic cage. An average of ten readings was taken for each animal. Heart rate were measured on the same day of blood pressure measurement. Body weights were recorded daily.

RT-PCR Southern blot analysis of human tissue kallikrein mRNA

Total RNA was extracted from fresh rat tissues by TRIZOL (BRL, Grand Island, NY). The reaction mixture for RT contained 1 μg of total RNA from several tissues, 20 pmol of the 3' primer (5'-GCCACAAGGGACGTAGC-3' located on the fifth exon of the human tissue kallikrein gene) (SEQ ID NO:4), 20 nmol of dNTP, 0.2 mmol of DTT, 4 μl of 5 x reverse transcription buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 200 U of Maloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) in a total volume of 20 μl. The reverse transcription reaction mixture was incubated at 37°C for 1 hr to synthesize the first strand of cDNA. Twenty pmol of 5' primer (5'-CATTTCAGCACTTTCCA-3' located on the second exon of the human tissue kallikrein gene) (SEQ ID NO:5), 5 μl of 10x PCR buffer and 0.5 U of Taq DNA polymerase were added to the RT mixture to a total volume of 50 μl followed by 30 cycles of hot-start PCR (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) with Ampliwax (Perkin-Elmer Cetus Instruments, Norwalk, CT) in a thermal cycler. Thirty μl of RT-PCR products were subjected to a Southern blot analysis. A specific oligonucleotide (5'-ACGACCTTCACAGCGTC-3' located on the third exon of the human tissue kallikrein gene) (SEQ ID NO:6) was used as a probe for hybridization at 42°C. The membrane was washed in 6x SSC twice at room temperature and exposed to X-Omat film at -80°C (Eastman Kodak Co., Rochester, NY).

Serum and urine collection

At various time points after injection of adenoviral vectors, serum was collected and measured for human tissue kallikrein levels. The 24 hr urine samples were collected on day 7 using metabolic cages. To eliminate the contamination of food particles in urine samples, rats only received tap water during the collection period. Urine samples were collected and centrifuged at 1000x g to remove particles. The

volume was measured and the supernatant was used for further analysis. Blood urea nitrogen was measured on day 10 by a modified urease-indophenol method (53).

Enzyme-linked immunosorbent assay (ELISA) for human tissue kallikrein

The level of human tissue kallikrein in serum was determined by ELISA (42). Briefly, a 96-well Microplate was coated with human tissue kallikrein immunoglobulin G (1 μg/ml in PBS, 100 μl/well) at 4°C overnight. The plate was blocked with 200 μl of PBS containing 1% bovine serum albumin at 37°C for 1 hr and washed three times with PBS containing 0.1% Tween-20. One hundred microliters of human tissue kallikrein standard (0.4 ng/ml to 25 ng/ml) or samples were added into each well. The plate was incubated at 37°C for 1.5 hr and washed. Biotin-labeled human tissue kallikrein immunoglobulin G was added to each well at a concentration of 1 μg/ml in a total volume of 100 μl. The plate was incubated at 37°C for 1 hr and washed. Avidin-peroxidase at a concentration of 1 μg/ml and 100 μl/well was added and incubated with the substrate solution (3 mg of 2,2'-azinobis (3-ethylbenzthiazoline) sulfuric acid, 10 μl of 3% H_2O_2 in 10 ml of 0.1M citric acid, pH 4.3). After 30 min, the plate was read on an ELISA plate reader at 414 nm for absorbance.

Urinary NOx (nitrite/nitrate) and kinin measurements.

Urine samples were sent to the New York Medical College for measurements of NOx content. Urinary NOx contents were measured by a calorimetric assay based on the Griess reaction (54). Urinary kinin levels were measured by a direct kinin radioimmunoassay as previously described (55).

Measurement of urine flow rate, glomerular filtration rate and renal blood flow.

Rats were anesthetized with pentobarbital (50 mg/kg, ip) and placed on a heating pad for maintenance of body temperature at 37°C. After tracheotomy, a cannula was placed in the jugular vein for infusion of fluids and drugs. A cannula was placed in the right femoral artery for the measurement of blood pressure and for blood sampling. The bladder was cannulated to allow urine collection from the right kidney. The left kidney was exposed by a flank incision, freed of perirenal tissue, placed in a Lucite cup and bathed in 0.9% NaCl and then the ureter was cannulated. Hydropenic

preparations were maintained by an intravenous injection of 1.2 ml of 0.9% NaCl containing 10% polyfructosan (Inutest, Laevosan-Gesellschaft, Estermannstraße, Austria) and 2% para-aminohippuric acid (PAH) (Merck Sharp & Dohme, West Point, PA) *via* the cannula in the jugular vein during the experimental period. Forty-five minutes was allowed for the preparation to reach a steady state. Timed urine collections were obtained, with blood (0.6 ml) collected between clearance periods. For maintenance of hematocrit, red blood cells from each blood sample were reconstituted to the same volume with 0.9% NaCl and reinjected through the arterial cannula. At the end of each experiment, kidneys were excised, blotted and weighed. Urine volume was determined gravimetrically. Polyfructosan and PAH concentrations were determined by modified anthrone and colorimetric methods, respectively (56). Glomerular filtration rate (GFR) and renal plasma flow (RPF) were determined from the clearance of polyfructosan and PAH, respectively. Renal blood flow (RBF) was calculated from RPF and hematocrit. Clearance data were normalized to kidney weight.

Morphological and histological investigation

Rats were anesthetized with pentobarbital (50 mg/kg body weight) and hearts and kidneys were removed, cleaned, washed in saline, blotted and weighed. The right ventricular free wall was carefully dissected from the left. The intraventricular septum was thus included in the left ventricular weight. Values from statistical analyses are reported as mean±SEM. Sections of the kidney and heart were preserved in 4% buffered formaldehyde solution and embedded in paraffin. Five μm-thick sections were cut and stained with hematoxylin-eosin and/or periodic acid-Schiff (PAS) and analyzed microscopically and morphometrically. For assessing the hypertrophy of myocardial cells, the shortest transverse diameter of fibers sectioned through the nuclei was measured with an ocular with an engraved measuring scale. An ocular micrometer was calibrated against a stage micrometer and conversion factors were calculated for low (4x objective) and high (45x objective) magnifications. The mean diameter of 400 cardiomyocytes in each group was measured with a calibrated eyepiece at a magnification factor of 450x. All sections were evaluated by independent personnel without the prior knowledge of the group in which the rats belonged.

Statistical analysis

Group data are expressed as mean±SEM. Statistical analysis was performed by ANOVA (analysis of variance) and Student's *t*-test. Differences were considered significant at a value of $P<0.05$.

Expression of human tissue kallikrein mRNA in rats receiving kallikrein gene delivery.

Human tissue kallikrein mRNA in tissues of rats receiving gene delivery was analyzed by RT-PCR followed by Southern blot analysis using specific oligonucleotide probes for human tissue kallikrein. Total RNAs were prepared from heart, liver, kidney, and aorta four days after intravenous injection of the adenoviral Ad.CMV-cHK carrying the human tissue kallikrein gene or the control adenovirus Ad.CMV-LacZ carrying the β-galactosidase gene under the control of the cytomegalovirus (CMV) promoter. Human tissue kallikrein mRNA was detected mainly in the liver and kidney and to a lesser extent in the heart and aorta. The expression of human tissue kallikrein mRNA was not detected in control rats receiving Ad.CMV-LacZ. Similar levels of β-actin mRNA were detected in tissues of rats injected with Ad.CMV-cHK or Ad.CMV-LacZ, indicating the integrity of RNA in these samples. The results show that human tissue kallikrein is expressed in rat tissues relevant to cardiovascular and renal function following gene transfer.

Time course of immunoreactive human tissue kallikrein in rats receiving kallikrein gene delivery. Immunoreactive human tissue kallikrein levels in rats receiving kallikrein gene delivery were measured by an ELISA specific for human tissue kallikrein. Human tissue kallikrein in serum and urine of rats receiving gene delivery displayed parallelism to the human tissue kallikrein standard, indicating their immunological identity. No immunoreactive human tissue kallikrein was detected in serum or urine of control rats receiving adenovirus Ad.CMV-LacZ. Following intravenous injection of adenovirus Ad.CMV-cHK, serum levels of immunoreactive human tissue kallikrein increased rapidly, reaching the highest level of 744±107 ng/ml (mean±SEM, n=5) at three days post gene delivery and declined gradually.

Decreased blood urea nitrogen in rats receiving kallikrein gene delivery.

At 10 days post gene delivery, the gentamycin-treated group with or without Ad.CMV-LacZ gene delivery had four times higher levels of blood urea nitrogen (BUN) than the control group given saline (85.4±0.6 and 85.4±1.2 vs. 19.0±0.3 mg/100 ml, n=4, $P<0.01$). The BUN levels (28.6±0.3 mg/100 ml serum, n=4) in rats injected with gentamycin and Ad.CMV-cHK were markedly reduced to those of control rats injected with only saline (19.0±0.3 mg/100 ml serum).

Effects of kallikrein gene delivery on the physiological parameters in gentamycin-induced nephrotoxicity in rats.

Table 6 shows the results of physiological analysis of gentamycin-treated rats seven days after gene delivery. There were no apparent changes in the body weight, blood pressure, heart rate, urine volume and water intake among the groups with gentamycin administration or between rats injected with Ad.CMV-LacZ vs. Ad.CMV-cHK. However, urinary kinin levels increased three-fold after kallikrein gene delivery as compared to Ad.CMV-LacZ group (78.4±24.1 vs. 26.5±4.5 ng/100 g body weight/day, n=3, $P<0.01$) in gentamycin-treated rats . Urinary human tissue kallikrein (3.2±0.9 μg/100 g body weight/day, n=3) can be detected in rats seven days post-kallikrein gene delivery but not in rats injected with Ad.CMV-LacZ.

Effects of kallikrein gene delivery on the renal function in gentamycin nephrotoxic rats.

Table 7 shows the results of renal hemodynamics in gentamycin nephrotoxic rats ten days after gene delivery. The kidney weights of rats treated with gentamycin with or without Ad.CMV-LacZ or Ad.CMV-cHK gene delivery is significantly higher than those of control rats injected with saline. Kallikrein gene delivery caused a significant increase in urine flow (18.5 ± 0.4 μl/min/g kidney weight, n=4, $P<0.01$) as compared to the group receiving control adenovirus Ad.CMV-LacZ (5.0 ± 0.2 μl/min/g kidney weight, n=4), gentamycin alone (4.4 ± 0.3 μl/min/g kidney weight, n=4) or control rats injected with saline (5.7 ± 0.4 μl/min/g kidney weight, n=4). Similarly, GFR (3.07 ± 0.07 vs. 0.83 ± 0.04 μl/min/g kidney weight, n=4, $P<0.01$) and RBF (32.0 ± 2.1 vs. 9.2 ± 0.6 μl/min/g kidney weight, n=4, $P<0.01$) were significantly increased

in rats injected with Ad.CMV-cHK compared to rats receiving the control adenovirus Ad.CMV-LacZ.

Protective effects of kallikrein gene delivery on cardiac hypertrophy in gentamycin nephrotoxic rats.

The mean left ventricular weight of the gentamycin-treated rats with or without Ad.CMV-LacZ increased significantly (0.34±0.02 g/100 g body weight, and 0.32±0.03 g/100 g body weight, n=4) as compared to that of rats injected with saline alone (0.26±0.01 g/100g body weight, n=4, $P<0.01$) and kallikrein gene delivery significantly reduced left ventricular weights (0.27±0.01 g/100 g body weight, n=4, $P<0.01$) to those of control rats. Similarly, the mean cardiomyocyte diameter of the gentamycin-treated group with or without Ad.CMV-LacZ increased significantly (13.9±0.1 μm, n=400 and 13.8±0.3 μm, n=400) as compared to that of rats injected with saline alone (11.7±0.5 μm, n=400, $P<0.01$) and kallikrein gene delivery significantly reduced cardiomyocyte size as compared with rats receiving Ad.CMV-LacZ (11.0±0.2 vs. 13.9±0.1 μm, n=400, $P<0.01$). These results show that somatic delivery of the human tissue kallikrein gene attenuates cardiac hypertrophy in this model of rats with nephrotoxicity.

Effects of kallikrein gene delivery on kidney morphology.

The morphology of sections of the kidney stained with hematoxylin and eosin (H&E) was examined with light-microscopy. The fixation, embedding and staining of non-treated control animals resulted in well preserved kidney morphology in both the cortex and medulla. Widespread areas of tubular dilation and damage were observed ten days after gene delivery in the renal cortex of rats injected with gentamycin and Ad.CMV-LacZ. Most proximal tubules were damaged and many were filled with necrotic cells. Distal tubules exhibited less damage and collecting ducts appeared relatively normal. Cortical renal tubular lumen were often filled with protein casts. Medullary tubules did not appear damaged in structure, but protein casts often filled the lumens, particularly in the outer medulla. In the kallikrein gene-treated group (Ad.CMV-cHK), swelling of proximal tubular cells was seen, but frank cellular necrosis was rare. Fewer dilated

renal tubules were observed after kallikrein gene delivery than in the Ad.CMV-LacZ group.

The morphology of kidney sections stained by periodic acid Schiff reaction (PAS) for carbohydrates of brush borders and basement membranes was also examined. The morphology of the kidney in control rats without gentamycin treatment was well preserved in both the cortex and medulla. In the renal cortex of rats injected with gentamycin and Ad.CMV-LacZ ten days after gene delivery, lumenal casts were generally PAS-positive, most probably derived from sloughed brush border glycoprotein of damaged proximal tubule cells. In the kallikrein gene-treated group (Ad.CMV-cHK), PAS staining of sections from these animals often showed intact brush borders on proximal tubule cells. Although protein casts were found in the outer medulla, they were not as large or as abundant as in the Ad.CMV-LacZ group. Glomeruli appeared relatively normal in the Ad.CMV-cHK group.

At 14 days post injection, renal damage was comparable to that seen after ten days of gentamycin treatment but was more wide-spread in the kidneys of the Ad.CMV-LacZ animals, in which tubular dilation and necrosis were very extensive. Indeed, few normal proximal tubules were observed and many protein casts were present in the outer medulla. By contrast, kidneys of animals of the kallikrein gene-treated group exhibited morphological indications of recovery. Renal tubular damage was not as severe as the damage seen in the Ad-CMV-LacZ group. Enlarged nuclei and mitotic structures were often observed in renal tubular cells, suggesting the likelihood of renal tubular regeneration. Few protein casts were present in the medulla. In all cases, despite the obvious tubular damage in all animals treated with gentamycin, the renal corpuscles (glomeruli and Bowman's capsule) exhibited little histological evidence of damage when stained with H&E.

This study demonstrates that adenovirus-mediated delivery of the human tissue kallikrein gene *via* intravenous injection displayed protective effects on

gentamycin-induced nephrotoxicity in rats. High levels of recombinant human tissue kallikrein were found in the rat blood stream and urine in a time-dependent manner following injection of the adenovirus carrying the human tissue kallikrein gene. Kallikrein gene delivery significantly attenuated proximal tubular damage: loss of brush border as well as frank cellular necrosis, and reduced protein casts of lumens of medullary tubules. In addition, kallikrein gene delivery also enhanced renal function and inhibited cardiac hypertrophy. The expression of human tissue kallikrein mRNA was identified in tissues relevant to cardiovascular and renal function such as the kidney, aorta and heart.

In addition to the morphological changes, significant increases in urine flow, GFR and RBF as well as a significant decrease in elevation of blood urea nitrogen levels were observed in gentamycin-treated rats after kallikrein gene delivery. These results showed that systemic delivery of the human tissue kallikrein gene had protective effects on renal function in this rat model of acute renal failure.

In conclusion, adenovirus-mediated human tissue kallikrein gene delivery has protective effects on nephrotoxicity and cardiac hypertrophy in rats with gentamycin administration. Although gentamycin-induced nephrotoxicity can be treated with drugs or dietary supplements such as fish oil or calcium (57), these treatments require daily administration. This study shows that kallikrein gene delivery attenuates renal injury as well as enhances renal function.

IV. Production of a nucleic acid comprising a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide and production of an adenoviral vector comprising a nucleic acid comprising a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide.

Full length cDNAs or genes encoding TK and ANP can be subcloned into a eukaryotic expression vector under the control of a CMV promoter or RSV 3'-

LTR promoter. The transcription unit, including the CMV or RSV promoter, TK and ANP gene or cDNA and an SV40 polyA sequence can be inserted into the Ad. link.1 adenovrial shuttle vector. After confirmation by DNA sequencing, the plasmid DNA containing the desired insert can be purified and used to co-infect human embryonic kidney cells 293 (ATCC accession number CRL 1573) with a recombinant-deficient adenovirus type 5 with an E1A deletion. The viral stocks containing the TK and ANP inserts can be monitored by ELISA and purified for amplification. The amplified viral stocks can be purified by CsCl gradient centrifugation and titered before use for *in vivo* transfection.

V. Administration of adenovirus containing nucleic acid encoding human tissue kallikrein and/or atrial natriuretic peptide to humans.

To treat a hypertension-associated or non-hypertension associated renal or cardiac disorder in a human, an initial dosage of 10^9 to 10^{10} pfu of adenovirus containing nucleic acid encoding TK and/or ANP is administered intravenously. In some instances, localized injection into the kidney is through a catheter inserted into the renal artery. In addition, a catheter inserted into the carotid artery is used for direct delivery of adenovirus to the heart. If additional injections are indicated, they can be administered at six month intervals.

The efficacy of treatment of a renal disorder is monitored at about ten days after gene delivery by adenovirus administration. Clinical parameters which can be monitored include renal sodium excretion, urine volume, urinary sediment and urine creatinine. Reduction of absence of proteinuria, edema, hematuria, azotemia and casts, as well as increased urine volume and sodium excretion are indicators of effective treatment.

The efficacy of treatment of cardiac disorder is monitored at about 20 days after gene delivery by adenovirus administration. Improvement in morphology and function as detected by electrocardiography, magnetic resonance imaging and positron emission tomography are indicators of effective treatment.

For treatment of restenosis, efficacy of treatment is monitored at about 20 days after gene delivery by angiocardiography.

VI. Atrial Natriuretic Peptide Gene Delivery Attenuates Gentamycin-Induced Nephrotoxicity in Rats

Preparation of replication-deficient adenovirus vector Ad.RSV-ANP.

Plasmid RSV-ANP was constructed as previous described (61), in which the expression of human ANP cDNA was under the control of the Rous sarcoma virus long terminal repeat (RSV-LTR) and was followed by a SV40 poly A signal sequence. The transcription unit of RSV-ANP-poly A, including the RSV-LTR, the human ANP cDNA, and a Simian virus 40 poly A signal sequence, was released from the RSV-ANP plasmid by Sal I digestion. Plasmid pAd.RSV-ANP was constructed by inserting the released fragment into the adenovirus shuttle vector pAdLink.1 at a Sal I site. The pAd.RSV-ANP plasmid DNA was purified using a Qiagen plasmid DNA kit (Qiagen, Chatsworth, CA). The purified DNA was sent to the Institute for Human Gene Therapy, Wistar Institute, Philadelphia to generate the adenovirus Ad.RSV-ANP harboring the RSV-ANP-poly A transcription unit. Final production of Ad.RSV-ANP and Ad.RSV-LacZ harboring the LacZ gene was carried out in this laboratory.

Animal treatment.

Sprague-Dawley rats (male, 200-220 g body weight, Harlan Sprague Dawley,Inc., Indianapolis, IN) were used in this experiment. Rats were housed at a constant room temperature with a 12 hr light/dark cycle and had free access to tap water and rat chow. The rats were divided at random into four groups. Three groups received gentamycin sulfate (Sigma Chemical, St. Louis, MO) subcutaneously at a dose of 80 mg kg in saline solution (0.9% NaCl). The injection was carried out for 10 consecutive days. The first group was injected with adenovirus Ad.RSV-ANP (1.2×10^{9} plaque-forming units) and the second group with same number of adenovirus Ad.RSV-LacZ via the tail vein on the first day of gentamycin treatment. Third group received gentamycin alone. The control

group was injected subcutaneously with saline. The animal experiments were conducted in a accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Southern blot analysis of ANP mRNA.

Total RNA was extracted from fresh rat tissues by TRIZOL (BRL, Grand Island, NY). The reaction mixture for RT contained 1 μg of total RNA from several tissues, 20 pmol of the 3' primer (5'-CACTGAGCACTTGTGGG-3' located at the second exon of the human ANP gene; SEQ ID NO:7), 20 nmol of dNTP, 0.2 mmol of DTT, 4μl of 5 x reverse transcription buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2) and 200 U of Maloney murine leukemia virus reverse transcriptase (BRL, Gaitherburg, MD) in a total volume of 20μl. The reverse transcription reaction mixture was incubated at 37°C for 1 hr to synthesize the first strand of cDNA. Twenty pmol of 5' primer (5'-CACCGTGAGCTTCCTCCTTT-3' from the first exon of the human ANP gene; SEQ ID NO:8), 5 μl of 10 x PCR buffer, and 0.5 U of Taq DNA polymerase were added to the RT mixture to a total volume of 50μl followed by 30 cycles of hot-start PCR (94°C, 1 min; 55°C, 1 minute; 72°C, 1 min) with Ampliwax (Perkin-Elmer Cetus Instruments, Norwalk, CT) in a thermal cycler. Thirty μl of RT-PCR products were subjected to a Southern blot analysis. A specific oligonucleotide (5'-TAGGTCAGACCAGAGCT-3' from the first exon of the human ANP gene; SEQ ID NO:9) was used as a probe for hybridization at 42°C. The membrane was washed in 3 x SSC twice at 42°C and exposed to X-Omat film at-80 °C (Eastman Kodak Co., Rochester, NY).

Serum and urine collection.

At various time points after injection of adenoviral vectors, serum was collected and measured for blood urea nitrogen (BUN) levels using a modified urease-indophenol method (62). Twenty-four-hour urine samples were collected on day 7 using metabolic cages. Urine samples were collected and centrifuged at

1000 x g to remove particles. The volume was measured and the supernatant was used for further analysis.

Tissue preparation.

At 10 days after gene delivery, one rat from each group was anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight and perfused with normal saline by cardiac puncture. Tissues were homogenized in normal saline with a Polytron (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 600 x g for 10 min. The supernatant was incubated in 0.5% sodium deoxycholate and then centrifuged at 10,000 x g for 30 min. Total protein in the supernatant was determined by Lowry's method (63).

Radioimmunoassay (RIA) for human ANP.

Tissue ANP levels were measured by a RIA for human ANP as previously described (61). Briefly, serial dilutions of standard ANP (10 - 1280 pg) or tissue extracts (100µl) were incubated with goat anti-human ANP antiserum (1:1500 dilution, Sigma Chemical Co., St. Louis, MO) in a solution containing 0.01M PBS, pH 7.4, 0.3% BSA, 0.1% Triton x100, 0.1 mM EDTA and 0.1% sodium azide, and ^{125}I-ANP tracer (10,000 cpm in 100 µl) in a total volume of 400 µl for 18-24 hr at 4°C. The reaction was stopped by adding 800µl of 25% polyethylene glycol (PEG) in PBS containing 0.1% sodium azide and 400 µl of 1% bovine gamma-globulin in PBS containing 0.1% sodium azide. The radioactivity of the precipitate was determined in a gamma counter.

Radioimmunoassay for cGMP.

Kidney tissues (100 mg) were homogenized in 1 ml 0.1 N HCl and the homogenatecentrifuged at 12,000 x g for 30 min. The supernatant was diluted to 1: 100 and the procedure for assay of cGMP was conducted according to the general procedure as previously described (64). Protein concentration was determined by Lowry's method (63).

Measurement of urine flow rate, glomerular filtration rate and renal blood flow.

Rats were anesthetized with pentobarbital (50 mg/kg, i.p) and placed on a heating pad for maintenance of body temperature at 37°C. After tracheotomy, a cannula was placed in the jugular vein for infusion of drugs. A cannula was placed in the right femoral artery for the measurement of blood pressure and for blood sampling. The bladder was cannulated to allow urine collection from the kidney. Hydropenic preparations were maintained by an intravenous injection of 1.2 ml of 0.9% NaCl containing 10% polyfructosan (Inutest, Laevosan, Linz, Austria) and 2% para-aminohippuric acid (PAH; Merck Sharp & Dohme, West Point, PA, USA) via a cannula in the jugular vein during the experimental period. Forty-five minutes was allowed for the preparation to reach a steady state. Timed urine collections were obtained, with blood (0.6 ml) collected between clearance periods. For maintenance of hematocrit, red blood cells from each blood sample were reconstituted to the same volume with 0.9% NaCl and reinjected through the arterial cannula. At the end of each experiment, kidneys were excised, blotted, and weighed. Urine volume was determined gravimetrically. Polyfructosan and PAH concentrations were determined by modified anthrone and colorimetric methods, respectively (65). Glomerular filtration rate (GFR) and renal plasma flow (RPF) were determined from the clearance of polyfructosan and PAH, respectively. Renal blood flow (RBF) was calculated from RPF and hematocrit. Clearance data were normalized to kidney weight.

Morphological and histological investigations.

Rats were anesthesized with pentobarbital (50 mg/kg, i.p) and kidneys were removed, cleaned, washed in saline, blotted and weighed. Sections of the kidney were preserved in 4% phosphate-buffered formaldehyde solution and embedded in paraffin. Fiveμm-thick sections were cut and stained with hematoxylin and eosin (H & E) and/or periodic acid-Schiff (PAS) and analyzed microscopically and morphometrically. Morphological changes were scored as follows: (1) proximal tubule damage was found in parts of the outer cortex; (2) proximal tubule damage was found throughout outer cortical nephrons; (3)

proximal tubule damage was found throughout outer and inner cortex; (4) proximal tubule damage was found throughout the cortex and extended into the outer medulla. A score of zero (found only in controls) indicated no observable proximal tubule damage. Morphological evidence of proximal tubule damage included: swelling and disruption of cells, cell sloughing and tubular casts, and loss of PAS-positive apical brush border. All sections were evaluated independently by individuals without the prior knowledge of the group to which the rats belonged.

Statistical analysis.

Group data are expressed as mean ± SEM. One- or two-way analysis of variance followed by a multiple means comparison test (ScheffÄ test) was used to compare the means of different groups. Differences were considered significant at a value of $P<0.05$.

Expression of human ANP mRNA in gentamycin-induced nephrotoxic rats.

Human ANP mRNA in gentamycin-induced nephrotoxic rats after gene delivery was analyzed by RT-PCR followed by Southern blot using specific oligonucleotide probes for human ANP. Total RNAs were prepared from heart, aorta, kidney, and liver at 4 days after intravenous injection of adenoviral vectors Ad.RSV-ANP or Ad.RSV-LacZ. ANP mRNA was detected in the kidney, heart, aorta and liver. The expression of ANP mRNA was not detected in control rats receiving adenoviral vector Ad.RSV-LacZ . Similar levels of β actin mRNA were detected in tissues of both experimental and control groups, confirming the integrity of RNA in these samples. The results show that human ANP is expressed in tissues relevant to cardiovascular and renal function following gene transfer in gentamycin-induced nephrotoxic rats.

Immunoreactive human ANP levels after gene delivery.

The levels of human ANP in gentamycin-induced nephrotic rats were analyzed by a RIA specific for human ANP. Immunoreactive human ANP was detected in the heart (480.0 ± 21.5 ng/mg protein) and kidney (95.4 ± 9.2 ng/mg

protein) at 4days after gene delivery. Linear displacement curves for immunoreactive ANP in the heart and kidney of gentamycin-induced nephrotic rats were parallel with the standard curve of human ANP, indicating their immunological identity. Serial dilutions of heart, liver and kidney extracts from control rats injected with Ad.RSV-LacZ showed a lack of parallelism with the human ANP standard curve. These results indicate that goat anti-human ANP antibody has some cross-reactivity with rat ANP; however, human and rat ANP are not immunologically identical and are distinguishable in the RIA.

Decreased blood urea nitrogen in rats receiving ANP gene delivery.

BUN levels in the gentamycin-treated group with or without Ad.RSV-LacZ gene delivery began to rise at 7 days after gentamycin administration. At 10 days post gene delivery there were 2 times higher levels of BUN in rats with gentamycin administration than the control group given saline (40.1 ± 0.3 and 39.0 ± 0.2 vs. 24.1 ± 0.1 mg/100 ml serum, n=5, $P<0.01$). Although the BUN levels in rats injected with gentamycin and Ad.RSV-ANP (33.3 ± 0.3 mg/100 ml serum, n=5) was significantly higher than the control group given saline, it was significantly lower than that of gentamycin-treated group with or without Ad.RSV-LacZ groups ($P<0.01$). The results indicate that ANP gene delivery significantly attenuated BUN elevation in gentamycin-induced nephrotoxicity in rats.

Effects of ANP gene delivery on physiological parameters in gentamycin-induced nephrotoxicity in rats.

There were no significant differences in all parameters between the control group and the gentamycin-treated groups with or without Ad.RSV-LacZ. However, ANP gene delivery caused a significant increase in urine volume (16.1 ± 1.3 vs. 8.9± 1.1 ml/day/100 g body weight, n=8, $P<0.01$) and water intake (17.5 ± 2.1 vs. 5.6 ± 1.0 ml/day/100 g body weight, n=8, $P<0.01$) compared to control rats receiving Ad.RSV-LacZ. Urinary sodium and potassium excretion in

the group receiving ANP gene delivery was also significantly higher than that of other groups.

Effects of ANP gene delivery on the renal function in gentamycin nephrotoxic rats.

In gentamycin-induced nephrotoxic rats at 10 days after gene delivery, ANP gene delivery caused a significant increase in urine flow (11.1 ± 0.3 ml/min/g kidney weight, n=4, P<0.01) as compared to the Ad.RSV-LacZ gene delivery group (5.0 ± 0.2 ml/min/g kidney weight, n=4), the gentamycin-treated group (4.4 ± 0.3 ml/min/g kidney weight, n=4), and the saline-injected control rats (5.7 ± 0.4 ml/min/g kidney weight, n=4). Similarly, rats injected with Ad.RSV-ANP induced significant increase in GFR (1.83 ± 0.05 vs. 0.83 ± 0.04 ml/min/g kidney weight, n=4, P<0.01) and RBF (18.9 ± 0.9 vs. 9.2 ± 0.6 ml/min/g kidney weight, n=4, P<0.01) when compared with rats receiving the control adenovirus Ad.RSV-LacZ.

Effects of ANP gene delivery on renal cGMP.

Ten days after gene delivery, cGMP levels were significantly lower in gentamycin-treated groups with or without Ad.RSV-LacZ than the control group received saline (1.3 ± 0.1 and 1.3 ± 0.1 vs. 2.8 ± 0.1 pmol/mg protein, n=4, P<0.01). However, a significant increase of cGMP in the kidney was observed in the Ad.RSV-ANP group (4.0 ± 0.1 pmol/mg protein, n=4, P<0.01) compared to the control Ad.RSV-LacZ group or control with saline.

Effects of ANP gene delivery on kidney morphology.

The fixation, embedding and staining of non-treated control animals resulted in well preserved kidney morphology in both the cortex and medulla. A widespread tubular dilation and damage were observed in the renal cortex of gentamycin-treated rats either alone or followed by Ad.RSV-LacZ. Most proximal tubules were damaged and many were either dilated or filled with necrotic cells. Distal tubules exhibited less damage and collecting ducts appeared relatively normal. Cortical renal tubular lumens were often filled with protein casts.

Medullary tubules did not appear damaged in structure, but protein casts often filled the lumens, particularly in the outer medulla. In the ANP gene-treated group (Ad.RSV-ANP), swelling of proximal tubular cells was seen, but fewer dilated renal tubules were present and frank cellular necrosis was rare.

The morphology of kidney stained by periodic acid Schiff reaction (PAS) for carbohydrates in brush borders and basement membranes was also examined. The morphology of the kidney in control rats without gentamycin treatment was well preserved in both the cortex and medulla. Proximal tubule brush border throughout cortex and outer medulla stained prominently with PAS, as did basement membrane in renal gromeruli and proximal tubules. In the renal cortex of rats injected with gentamycin alone or gentamycin and Ad.RSV-LacZ, there were dilated proximal tubules generally lacking of brush border. Lumenal casts were generally PAS-positive, most probably derived from sloughed brush border glycoprotein and reabsorption droplets from damaged proximal tubule cells. The ANP gene-treated group exhibited less damage than either gentamycin alone or gentamycin with Ad.RSV-LacZ group. Although some tubular damage persisted, evidence of preservation and regeneration of proximal tubules included: intense PAS staining of the apical brush border in many tubules and fewer protein casts thoughout the kidneys.

The effect of ANP gene delivery on morphological changes of the kidney was assessed in PAS-stained sections. The quantitative evaluation of renal tubular damage was scored as described herein. Gentamycin-treated animals with or without Ad.RSV-LacZ had the most extensive damage (Score; gentamycin, 3.0 ± 0.3; gentamycin and Ad.RSV-LacZ; 2.7 ± 0.3; n=8) and therewere no differences between these two groups. Although the rats receiving gentamycin and Ad.RSV-ANP had some tubular damage (Score; 1.3 ± 0.1, n=8), the damage was significantly less than in two control groups ($P<0.01$). The results indicate that ANP gene delivery significantly attenuated tubular damage induced by gentamycin.

VII. Adenovirus-Mediated Kallikrein Gene Delivery Reduces Aortic Thickening and Stroke-Induced Death Rate in Dahl Salt-Sensitive rats

Animals

Dahl salt-sensitive rats (Male, 4 weeks old) from (Sprague-Dawley Harlan, Indianapolis, IN) were used in this study. Rats were divided into two groups. The first group was fed a standard rat chow (0.4% NaCl) and the other group was fed a high salt diet (4% NaCl) (Harlan Teklad, Madison, WI). All rats had free access to water. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD).

Preparation of replication-deficient adenovirus vector Ad.CMV-cHK

Plasmid CMV-cHK was constructed as described herein. In this construct, the expression of human tissue kallikrein cDNA was under the control of the cytomegalovirus (CMV) enhancer/promoter and was followed by a bovine growth hormone (BGH) poly A signal sequence. The purified DNA was sent to the Institute for Human Gene Therapy, Wistar Institute, Philadelphia for generation of adenovirus Ad.CMV-cHK harboring the CMV-cHK-poly A transcription unit. Adenovirus harboring the LacZ gene under the control of the CMV enhancer/promoter (Ad.CMV-LacZ) was purchased from the Institute for Human Gene Therapy.

Intravenous Delivery of Adenoviral Vectors

DS rats in the group fed a high salt diet containing 4% NaCl for 4 weeks were randomly divided into three groups and were intravenously injected with either Ad.CMV-cHK (n=13), Ad.CMV-LacZ (n=6) at a dosage of 2.4×10^{10} pfu (plaque formation unit) per rat or with saline through the tail vein. Six DS rats on a 4% NaCl diet did not receive any adenovirus injection.

Blood pressure measurement

The systolic blood pressure of rats was measured with a manometer tachometer (Nastume KN-210; Nastume Seisakusho Co. Ltd., Tokyo, Japan) using the tail-cuff method (67). An average of ten readings was taken for each animal after they became acclimated to the environment.

Urine collection and analysis of physiological parameters

Twenty-four-hour urine was collected from rats in metabolic cages at 5 and 11 days post gene delivery. In order to eliminate contamination of urine samples, animals received only water during the 24-hour collection period. Urine was collected and centrifuged at 1,000 x g to remove particles. The volume of the supernatant was measured and the samples were used for further analysis.

Tissue preparation

At the end of the experiment, rats were sacrificed. Brain was removed and homogenized to examine kallikrein activity. Tissue extracts were prepared as previously described. Total protein in the supernatant was determined by Lowry's method (68).

Enzyme-linked immunosorbent assay (ELISA) for human tissue kallikrein

Tissues were immersed in PBS (phosphate-buffered saline; pH 7.0) and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY). The levels of immunoreactive human tissue kallikrein in rat urine, and sera were determined by an ELISA specific for human tissue kallikrein as previously described (67).

Enzymatic assays toward low molecular weight kininogen substrates.

Canine low molecular weight kininogen was isolated according to the method of Johnson et al. (69). Kinin-releasing activities were measured by incubating rat aortic extracts (92) with canine low molecular weight kininogen (1.5) in 0.1 M sodium phosphate (pH 8.5) in a total volume of 500 at 37°C for 30 min. The reactions were stopped by boiling for 20 min. Released kinin was

assayed by a kinin RIA (70). Kininogenase activity is expressed as of kinin released per mg protein per 30 min.

Radioimmunoassay (RIA) of urinary kinin and cGMP

Urinary kinin levels were determined by a kinin radioimmunoassay as described (70). The procedure for assay of cyclic guanosine monophosphate (cGMP) was conducted as described (71).

Monitoring stroke development

The rats were monitored daily for the occurrence of stroke. The symptoms associated with stroke development have been previously described in SHRSP (72, 73) but not in DS rats. Initially, SHRSP develop convulsive repetitive forearm movement followed by inappropriate posture. In this study, stroke was often associated with lethargy and poor grooming. There is no fixed period between onset of the first behavioral symptoms of stroke and death. Some animals died abruptly after the first behavioral symptoms of stroke. Some animals were killed later, at a point when death was judged likely to occur within a day.

Morphological and histological investigations

Tissue sections were preserved in 4% buffered formaldehyde solution and embedded in paraffin. Five µm-thick sections were cut, stained with hematoxylin-eosin (H&E) and analyzed microscopically and morphometrically. Measurements of the thickness of aortic wall were also performed. Ten measurements taken from different position of each aorta were averaged. All sections were evaluated by independent personnel with no prior knowledge of the group from which the rats were obtained.

Statistical analysis

Data were analyzed using standard statistical methods. Repeated blood pressure measurements were taken after gene delivery for comparison between control and experimental groups at each time point with the use of ANOVA and

Fisher's protected least significant differences. Group data were expressed as mean ± SEM. Values were considered significantly different at a value of $P<0.05$.

Blood pressure reduction after intravenous injection of Ad.CMV-cHK

DS rats (4 weeks old) were fed a high salt (4% NaCl) diet or normal rat chow (0.4% NaCl) for 4 weeks until blood pressure differences between these two group exceeded 50 mmHg. DS rats on the high salt diet were divided into three groups. Two groups were intravenously injected with either Ad.CMV-cHK or Ad.CMV-LacZ through tail vein. One group was only injected with the vehicle, saline. Blood pressure was monitored weekly for DS rats fed a normal salt diet (0.4% NaCl) or a high salt diet (4% NaCl) from 1 to 4 weeks post gene delivery. Intravenous delivery of the human tissue kallikrein gene caused a significant reduction in blood pressure within 1 week after injection. A maximal blood pressure reduction of 21 mmHg was observed 14 days after gene delivery (201 ± 3 vs. 221 ± 8 mmHg, mean ± SEM, $n=7$, $P<0.01$). In contrast, the blood pressure of control rats on a normal salt diet (0.4% NaCl) remained at 135-155 mmHg throughout the experimental period.

Time-dependent expression of human tissue kallikrein after gene delivery

Following intravenous injection of Ad.CMV-cHK, immunoreactive human tissue kallikrein levels in rat sera were measured by ELISA. The highest level of recombinant human tissue kallikrein (967 ± 4 ng/ml, mean ± SEM, $n=4$) was observed on the fourth day after gene delivery. Human tissue kallikrein levels persisted for 4-5 weeks and decreased gradually from day 6 to day 24.

Kallikrein activities in the brain extracts site after gene delivery.

Kininogenase activity increased 2.8-fold in rat brain after kallikrein gene delivery (3.6 ± 0.6 ng of kinin released mg protein/30 min) compared to control rats receiving Ad.CMV-LacZ (1.3 ± 0.1 ng of kinin released/mg protein/30 min) (mean ± SEM, $n=4$, $P<0.05$), while the kininogen activity of DS rats on a 0.4% NaCl diet was 1.4 ± 0.2 ng of kinin released mg protein/30 min.

Effect of Ad.CMV-cHK gene delivery on urinary kinin and cGMP levels.

Following kallikrein gene delivery, urinary kinin levels increased 3.5-fold as comparing the rats receiving control virus Ad.CMV-LacZ (43.5 ± 8.4 vs. 12.2 ± 3.9 ng/day/100 g BW, mean ± SEM; n=6, P<0.05). Kinin levels of DS rats on a 0.4% NaCl diet was 9.8 ± 2.7 ng/day/100 g BW (n=6). Urinary cGMP levels alsoincreased significantly in rats receiving kallikrein gene delivery as compared to the Ad.CMV-LacZ group (13.51 ± 0.41 vs. 8.45 ± 0.41 nmol/day/100g BW, mean ± SEM; n=7, P<0.05). cGMP levels of DS rats on a 0.4% NaCl diet was 6.9 ± 1.4 nmol/day/100g BW (n=7).

Mortality rate of DS rats with stroke.

DS rats began to show symptoms of stroke, including lethargy, poor grooming, convulsive repetitive forearm movement, and/or hemiplegia, at five and a half weeks after high salt diet loading. Some rats died rapidly after the first behavioral symptoms of stroke. At 5 weeks after kallikrein gene delivery (62 days after high salt loading), a doubling of the survival rate was observed in hypertensive DS rats. The salt-induced mortality rate in DS rats receiving kallikrein gene delivery 5 weeks post injection was 27% (n=13) while 54% of DS rats (n=12) died from stroke with or without control adenovirus injection.

Effect of kallikrein gene delivery on salt-induced aortic thickening in DS rats.

The effect of kallikrein gene delivery on salt-induced aortic thickening in DS rats was determined as described herein. The thickness of the aortic wall was significantly reduced in the Ad.CMV-cHK group at 5 weeks post gene delivery (134.9 ± 1.7 vs.161.3 ± 1.3 (m, mean ± SEM, n=5, P<0.01) as compared to that of the Ad.CMV-LacZ group, while aortic wall thickness for DS rats on a 0.4% NaCl diet was 114.8 ± 1.5 (m (n=5). These results indicate that human tissue kallikrein gene delivery can attenuate, at least in part, salt-induced aortic hypertrophy in DS rats.

VIII. Kallikrein Gene Transfer Inhibits Vascular Smooth Muscle Cell Growth and Neointima Formation in Rat Artery after Balloon Angioplasty.

Animal treatment.

Local gene delivery: Male Sprague-Dawley rats (weight, 400-450 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a 2F embolectomy balloon catheter was introduced into the left common carotid artery via the external carotid artery. The balloon was inflated with sufficient saline to distend the common carotid and was then pulled back to the external carotid artery. This procedure was repeated three times and the catheter was then removed. After balloon injury of the left common carotid artery, the injured distal segment was isolated by temporary ligatures. The adenoviral particles of Ad.CMV-cHK or Ad.CMV-LacZ (2×10^9 pfu in 20 µl) were infused into the distal injured segment and incubated for 15 min at room temperature. After incubation, the cannula was removed and blood flow to the common carotid artery was restored. To investigate the potential kinin-mediated effect following kallikrein gene delivery, icatibant (Hoe 140), a specific antagonist for B2 receptor was infused intraperitoneally at a rate of 70 µg/kg/day via osmotic minipumps (Alzet 2ML2, Alza Corp. Palo Alto, CA) immediately post balloon angioplasty and Ad.CMV-cHK infusion. At 4 days after gene delivery, rats of each group were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused with saline through the ascending aorta. The liver, kidney, left and right common carotid artery, thoracic artery, and heart were rapidly isolated for RNA extraction. At 7 and 14 days after gene delivery, rats of each gene delivery group were anesthetized and artery and other tissues were isolated for protein measurements, RNA extraction, or morphometric analysis.

Systemic gene delivery: Male Sprague-Dawley rats (weight, 250-300 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a 2F embolectomy balloon catheter (Baxter Healthcare Corp.) was passed into the aorta via the femoral artery and placed distal to the renal artery. The balloon was inflated with sufficient saline and withdrawn slowly to the aortic bifurcation. This

procedure was repeated three times. After the surgery, rats were injected with 2 x 10^{10} plaque-forming units (pfu) of adenovirus containing the human tissue kallikrein gene (Ad.CMV-cHK) or control virus carrying the LacZ gene (Ad.CMV-LacZ) via the tail vein. At 4 days after gene delivery, rats of each group were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and perfused with saline through the ascending aorta. The liver, kidney, abdominal aorta, and heart were rapidly isolated for RNA extraction. At 7 and 14 days after gene delivery, rats of each gene delivery group were anesthetized and aorta was isolated for protein measurements, kininogenase assay, RNA extraction or morphometric analysis.

Preparation of adenovirus carrying the human tissue kallikrein gene.

Adenovirus containing the human tissue kallikrein gene, Ad.CMV-cHK was generated as previously described (74). Large quantities of high-titered adenovirus, Ad.CMV-cHK and Ad.CMV-LacZ, were prepared and purified for gene delivery (75).

Primary aortic smooth muscle cell culture.

Rat primary vascular smooth muscle cells (VSMC) were isolated from thoracic aorta of male Sprague-Dawley rats (200-250g) by the explant method (76). VSMC were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO/BRL-Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin sulfate. The cells exhibited a "hill and valley" growth pattern and were characterized by positive immunostaining with monoclonal antibodies against smooth muscle β actin (77). Cells were used between passages 3 and 10.

Adenovirus-mediated kallikrein gene transfer to VSMC.

Rat VSMC were subcultured into 24-well plates and the effect of adenovirus-mediated kallikrein gene transfer on cell proliferation was tested at 60-80% confluence. After growth-arrested for 48 hours, VSMC were transiently infected at 37°C with adenovirus (Ad.CMV-cHK or control virus Ad.CMV-LacZ)

(100 pfu/cell) in Dulbecco's Modified Eagle Medium (DMEM) for 6 hours under humidified air containing 5% CO_2 and cells were then washed with phosphate-buffered saline (PBS) and incubated with DMEM for cell proliferation study. Cultured medium was collected daily from 1 to 6 days after gene transfer for analyzing human kallikrein levels by ELISA. Cell pellets were extracted for total protein measurements.

[^{3}H] thymidine incorporation.

VSMC were seeded in 24-well plates and cultured in DMEM containing 10% fetal bovine serum at 37°C under humidified air containing 5% CO_2. Four replicates were used for each sample. At 4 days after transfection with Ad.CMV-cHK or Ad.CMV-LacZ, cells were incubated with 1.0 μCi/ml [^{3}H] thymidine for 4 hours in DMEM. At the end of incubation, cells were washed 3 times with PBS, precipitated with 10% trichloroacetic acid at 4°C for 30 min, washed 2 times with 95% ethanol and solubilized with 0.25 M NaOH plus 0.1% SDS. After neutralization with 1 M acetic acid, the radioactivity was determined with a liquid scintillation counter (Packard, Downers Grove, IL).

Enzymatic assays toward low molecular weight kininogen substrates.

Canine low molecular weight kininogen was isolated according to the method of Johnson et al. (78). Kinin-releasing activities were measured by incubating rat aortic extracts (10 μg) with canine low molecular weight kininogen (3 μg) in 0.1 M sodium phosphate (pH 8.5) in a total volume of 500μl at 37°C for 30 min. The reactions were stopped by boiling for 20 min. Released kinin was assayed by a kinin RIA (79). Kininogenase activity is expressed as μg kinin released per mg protein per 30 min.

Reverse transcription-polymerase chain reaction (RT-PCR) Southern blot analysis.

Total RNA was extracted with TRIzol(r) reagent according to the protocol recommended by the manufacturer (BRL, Gaithersburg, MD). Semi-quantitative RT-PCR Southern blot analyses were employed to determine the abundance of

bradykinin B1 and B2 receptors, rat tissue kallikrein, high/low-molecular weight kininogens, and T-kininogen mRNAs in non-treated, sham-operated, and injured carotid artery or injured-abdominal aorta after angioplasty. The expression of human tissue kallikrein in rat tissues after adenovirus-mediated gene delivery was examined by RT-PCR Southern blot analysis (74). Specific 5' primers and 3' primers used for RT-PCR and specific internal oligonucleotide probes for Southern blot analyses for human tissue kallikrein, rat tissue kallikrein, rat bradykinin B1 and B2 receptors, rat high/low-molecular weight kininogens and rat T-kininogen are shown below.

Rat tissue kallikrein:

5'-primer: 5'-TAC TAC TTC GGC GAA TAC CTA-3' (SEQ ID NO:10)

3'-primer: 5'-TCC AAT CCG TCA GGT GTG ATG-3' (SEQ ID NO:11)

probe: 5'-GAC GAC CTG GGG ACG ACT-3' (SEQ ID NO:12)

B1 receptor:

5'-primer: 5'-AAG ACA GCA GTC ACC ACT-3' (SEQ ID NO:13)

3' primer: 5'-CCG ATC TGG TGT TTG TC-3' (SEQ ID NO:14)

probe: 5'-AAG ACT GGG ACC TGC TGT AT-3' (SEQ ID NO:15)

B2 receptor:

5' primer: 5'-CCG TCT GGA CCT CCT TGA AC-3' (SEQ ID NO:16)

3' primer: 5'-GCT GAG GAC AAA GAT GTT C-3' (SEQ ID NO:17)

probe: 5'-TAC TCC TTC ATG GTC CGG AAC ACC A-3' (SEQ ID NO:18)

High/low molecular weight kininogen:

5' primer: 5'-GCC ACC CAG CTC TGC AAT AT-3' (SEQ ID NO:19)

3' primer: 5'-CTG CCC TTG TCA TCA CAT GA-3' (SEQ ID NO:20)

probe: 5'-TGT CAC GGT TGA AGC TT-3' (SEQ ID NO:21)

T-kininogen:

5' primer: 5'-GCC ACC CAG CTC TGC AAT AT-3' (SEQ ID NO:22)

3' primer: 5'-CTG CCC TTG TCA TCA CAT GA-3' (SEQ ID NO:23)
probe: 5'-TTC TTG TAC TCG CAC CA-3' (SEQ ID NO:24)

Human tissue kallikrein:
5' primer: 5'-CAT TTC AGC ACT TTC CA-3' (SEQ ID NO:25)
3' primer: 5'-GCC ACA AGG GAC GTA GC-3' (SEQ ID NO:26)
probe: 5'-ACG ACC TTC ACA GCG TC-3' (SEQ ID NO:27)

Signals were detected by autoradiography at -80°C and scanned into Adobe Photoshop 2.5 with the Hewlett Packard Scan Jet IICX/T.

Assays for cAMP and cGMP levels.

At one week after gene delivery, rats were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight and perfused with normal saline (0.9% NaCl) by cardiac puncture. Abdominal aorta was dissected and homogenized in 10 volumes of 0.1 N HCl with a Polytron (Brinkmann Instruments, Westbury, NY) at 4°C. The homogenate was centrifuged at 15,000 x g for 30 min and supernatants were stored at -80°C for assays. cAMP and cGMP levels were measured by RIA and protein concentrations were determined by Lowry's method as previously described (80).

Morphometric analysis.

At two weeks after gene delivery, rats were anesthetized and a catheter was placed in the ascending aorta and right atrium was cut open. The vascular system was perfused with saline and then perfusion-fixed with 4% formaldehyde at a perfusion pressure of 100mmHg. The left and right carotid artery were removed and embedded in paraffin. Each artery was divided into three segments that were separately embedded in paraffin. Cross-section rings (4 μm) were cut from each segment and stained with hematoxylin and eosin. The slides were photographed with a microscope at a magnification of 100 x. The lumen, neointima, media areas were traced and measured by using NIH Image 1.61 software package.

Statistical analysis.

Group data are expressed as mean ± SEM. Intima and media areas, intima/media area ratios were compared between experimental groups by one-way analysis of variance (ANOVA). Differences between kallikrein and control groups suggested by the ANOVA were further evaluated by Fisher's protected least-squares differences. Differences were considered significant at a value of $P<.05$.

Inhibition of primary cultured vascular smooth muscle cell growth by adenovirus-mediated kallikrein gene transfer.

Expression of recombinant human tissue kallikrein in primary cultured rat vascular smooth muscle cells (VSMC) was measured from 1 to 6 days after transfection with adenovirus carrying the human tissue kallikrein gene. Recombinant human tissue kallikrein level achieved the highest level, 310 ± 39 ng/ml (n=3) at 2 days after gene transfer and decreased to 135 ± 8 ng/ml (n=3) at 6 days after gene delivery. Expression of the human tissue kallikrein transgene in VSMC caused a significant inhibition in cell proliferation as measured by [^{3}H]thymidine incorporation into DNA at 4 days after gene transfer. The growth rate of VSMC transfected with Ad.CMV-cHK (2568 ± 198 cpm/well) was reduced to 59% of control cells with or without transfection with Ad.CMV-LacZ (4745 ± 329, or 4343 ± 120 cpm/well) (n=4, $P<.01$).

Differential expression of vascular tissue kallikrein-kinin system components after balloon angioplasty.

The expression of endogenous tissue kallikrein-kinin system components in rat blood vessels was analyzed with RT-PCR followed by Southern blot analysis using three gene-specific oligonucleotides for each transcript as described herein. At 1 and 2 weeks after balloon angioplasty via common carotid artery, the relative level of rat tissue kallikrein mRNA was markedly reduced when compared to control sham-operated rats. In contrast, bradykinin B1 receptor mRNA level increased at 1 week but was not changed 2 weeks after angioplasty.

No changes in high/low molecular kininogens, T-kininogen and bradykinin B2 receptor mRNA expression were observed in injured carotid artery. Similar levels of β actin were detected in both sham-operated and angioplasty groups, indicating that the RNA quality of these samples are internally consistent. A similar pattern in the differential expression of rat tissue kallikrein, bradykinin B1 and B2 receptors, and kininogens was observed in rat abdominal aorta after balloon angioplasty through the femoral artery.

Kallikrein activities at the injured site after gene delivery.

At 1 week after balloon injury, the relative levels of tissue kallikrein mRNA was markedly reduced while bradykinin B1 receptor mRNA increased when compared to sham-operated rats. The endogenous rat tissue kallikrein activities after balloon angioplasty (μg kinin released/mg protein/30 min) was reduced to a certain percentage of that in sham-operated rats (μg kinin released/mg protein/30 min) ($n=4$, $P<.01$) consistent with the suppression of endogenous rat tissue kallikrein gene expression at the injured vessels. Adenovirus-mediated kallikrein gene delivery significantly increased kinin-releasing activity in rat aorta after balloon angioplasty. Kininogenase activity increased 3.8-fold in rat aorta after kallikrein gene delivery (9.33 ± 2.00 μg kinin released/mg protein/30 min) compared to injury plus infection with control adenovirus Ad.CMV-LacZ (2.44 ± 0.33 μg kinin released/mg protein/30 min) (mean ± SEM, $n=3$, $P<.01$).

Expression of human tissue kallikrein mRNA in balloon-injured rat artery after gene delivery.

At 4 days after local administration of Ad.CMV-cHK into the balloon-injured left common carotid artery, human tissue kallikrein mRNA was only detected in the injured left carotid artery but not in the control right carotid artery, aorta, heart, liver or kidney by RT-PCR Southern blot analysis. Human tissue kallikrein mRNA was not detected in rats receiving Ad.CMV-LacZ. Similar levels of β actin were detected in both experimental and control groups, indicating the integrity of RNA in these samples. At 4 days after systemic delivery

of Ad.CMV-cHK via tail vein, human tissue kallikrein mRNA was detected in the aorta. as well as in the liver, kidney, and heart.

Adenovirus-mediated human tissue kallikrein gene transfer inhibited neointima formation.

To assess the *in vivo* effects of kallikrein gene delivery on vascular cell proliferation *in vivo*, balloon angioplasty was used to induce vascular injury. In this model, a consistent neointima formation developed within the first 2 weeks after balloon angioplasty. To assess the effect of kallikrein gene transfer, Ad.CMV-cHK or control virus Ad.CMV-LacZ were delivered at the time of balloon injury and the vessels were harvested 2 weeks after local gene delivery. Adenovirus-mediated kallikrein gene delivery significantly suppressed neointima formation in injured carotid artery post angioplasty as compared to rats injected with saline or with control virus carrying the LacZ gene.

Icatibant significantly blocks the protective effect of kallikrein gene delivery.

Kallikrein gene delivery significantly suppressed neointima formation in rat carotid artery (cross-sectional area: 85.9 ± 47.1 μm^2) when compared to control rats with or without injury plus infection with control adenovirus, Ad.CMV-LacZ (129.5 ± 10.4 μm^2, mean ± SEM, n=8, P<.01). There is a 39% reduction in intima/media ratio in rats receiving kallikrein gene delivery as compared to rats receiving control virus (0.80 ± 0.06 vs. 1.32 ± 0.10, mean ± SEM, n=8, P<.01). Reduction of neointima formation after kallikrein gene delivery was significantly blocked by icatibant (85.9 ± 7.1 μm^2 vs. 130.5 ± 6.5 μm^2, mean ± SEM, n=5, P<.01). No statistical difference was found among injured carotid artery after angioplasty with or without infected with Ad.CMV-LacZ and Ad.CMV-cHK with icatibant infusion.

Similarly, systemic kallikrein gene delivery significantly suppressed neointima formation in rat aorta (cross-sectional area: 92.1 ± 3.8 μm2) when compared with that in aorta exposed to either injury alone or injury plus infection

with control adenovirus, Ad.CMV-LacZ (135.0 ± 5.5 μm^2, mean ± SEM, n=7, P<.01). There is a 35% reduction in intima/media ratio in rats receiving kallikrein gene delivery as compared to rats receiving Ad.CMV-LacZ (0.88 ± 0.03 vs. 1.34 ± 0.06, mean ± SEM, n=8, P<.01). No statistical difference was found between injured abdominal aorta after angioplasty with or without infected with Ad.CMV-LacZ.

Cyclic AMP and cGMP levels in rat aorta after kallikrein gene delivery.

Cyclic AMP levels increased 5- to 8-fold in rat aorta after systemic delivery of the kallikrein gene (3.3 ± 1.2 pmoles/mg protein) compared to controls with or without Ad.CMV-LacZ infection (0.5 ± 0.2 and 0.6 ± 0.1 pmoles/mg protein, mean ± SEM, n=3, P<.05). cGMP levels in rat aorta increased more than 40-fold after kallikrein gene delivery (40.5 ± 17.9 pmoles/mg protein) compared to controls with or without Ad.CMV-LacZ infection (0.68 ± 0.31 and 0.98 ± 0.39 pmoles/mg protein, mean ± SEM, n=3, P<.05).

Example IX. Adenovirus-Mediated Kallikrein Gene Delivery Reverses Salt-Induced Renal Injury in Dahl Salt-Sensitive Rats

Animal treatment.

Dahl salt-sensitive rats (male, 4 weeks old) were purchased from Sprague-Dawley Harlan, Indianapolis, Indiana. Rats were divided into three groups. The control group was fed a standard rat chow (0.4% NaCl) (Harlan Teklad, Madison, WI). The experimental groups were fed throughout the study with a high salt diet (4% NaCl) (Harlan Teklad). All rats had free access to water. Throughout the study period, all animals were housed in a room that was kept at constant temperature (25 ± 1°C) and humidity (60 ± 5%) and was lighted automatically from 8:00 am to 8:00 pm. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD).

Preparation of replication-deficient adenoviral vector Ad.CMV-cHK.

Adenovirus vector Ad.CMV-cHK was prepared as previously described [81] in which the expression of human tissue kallikrein cDNA was under the control of the cytomegalovirus (CMV) enhancer/promoter and was followed by a bovine growth hormone (BGH) poly A signal sequence. Adenovirus harboring the LacZ gene under the control of the CMV enhancer/promoter (Ad.CMV-LacZ) was purchased from the Institute for Human Gene Therapy, Wistar Institute, Philadelphia.

Intravenous delivery of adenoviral vectors Ad.CMV-cHK and Ad.CMV-LacZ.

Seven Dahl-SS rats from experimental groups, which were fed a high salt diet containing 4% NaCl, were intravenously (IV) injected with either Ad.CMV-cHK or Ad.CMV-LacZ at a dosage of 1.2×10^{10} pfu (plaque formation units) per rat through the tail vein. During the experimental period, blood was collected daily from the tail vein after injection. Rat serum samples were frozen at -80°C until the expression level of immunoreactive human tissue kallikrein could be determined.

Blood pressure measurement.

The systolic blood pressure of rats was measured with a manometer-tachometer (Nastume KN-210; Nastume Seisakusho Co. Ltd., Tokyo, Japan) using the tail-cuff method [82]. Unanesthetized rats were introduced into a plastic holder mounted on a thermostatically controlled warm plate, which was maintained at 33-35°C during measurements. An average of ten readings was taken for each animal after they became acclimated to the environment.

Urine collection and analysis of physiological parameters

Twenty-four-hour urine of rats was collected in metabolic cages at 7 days post gene delivery. Rats were fed a 4% NaCl diet for 3 hours before placing them in metabolic cages that were supplied with drinking bottles. In order to eliminate contamination of urine samples, animals received only water during the 24-hour collection period. Urine was collected and centrifuged in a microfuge at 1,000 x g

to remove particles. The volume of the supernatant was measured and stored at -20°C until analysis for kinin, NOx, cyclic AMP (cAMP) and cyclic GMP (cGMP) and human tissue kallikrein levels.

Tissue preparation.

At the end of the experiment, all rats were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight. Blood samples were collected by direct cardiac puncture and chilled at 4°C overnight. The blood samples were centrifuged at 1,000 x g for 20 min and sera were removed and frozen at -20°C. At the same time, rats were perfused with normal saline (0.9% NaCl) via the heart. The whole heart, left ventricle, left and right kidneys were removed, blotted and weighed. Tissues of interest were removed and total RNAs were extracted by the trizol method (BRL, Gaitherburg, MD). The extracted RNA was quantified spectrophotometrically by absorbance at 260 nm, dissolved in diethyl pyrocarbonate-treated water, and stored at -80°C for further use.

RT-PCR Southern blot analysis of human tissue kallikrein mRNA.

RT-PCR Southern blot analysis using specific oligonucleotide probes for human tissue kallikrein (5'-primer, 5'-AACACAGCCCAGTTTGT-3', SEQ ID NO:28; 3' primer, 5'-CTTCACATAAGACAGCA-3', SEQ ID NO:29; internal probe, 5'-GACCTCAAAATCCTGCC-3', SEQ ID NO:30) was performed as previously described [81].

Enzyme-linked immunosorbent assay (ELISA) for human tissue kallikrein.

The levels of immunoreactive tissue kallikrein in rat serum and urine were measured by an ELISA specific for human tissue kallikrein as previously described [81]. Human tissue kallikrein standard ranged from 0.4 to 25 ng/ml. Since the antibody only recognizes active kallikrein [81], the immunoreactive kallikrein levels determined by ELISA represent active kallikrein.

Assays of urinary kinin, cGMP and cAMP levels.

Urinary kinin levels were determined by a direct kinin RIA as described [81]. The assays for cGMP and cAMP were conducted according to previously described procedures [83,84].

Measurement of glomerular filtration rate and renal blood flow.

Rats were anesthetized with pentobarbital (50 mg/kg, ip) and placed on a heating pad for maintenance of body temperature at 37°C. After tracheotomy, a cannula was placed in the jugular vein for infusion of fluids and drugs. A cannula was placed in the right femoral artery for the measurement of blood pressure and for blood sampling. The bladder was cannulated to allow urine collection from the right kidney. The left kidney was exposed by a flank incision, freed of perirenal tissue, placed in a Lucite cup, and bathed in 0.9% NaCl and then the ureter was cannulated. Hydropenic preparations were maintained by an intravenous injection of 1.2 ml of 0.9% NaCl containing 10% polyfructosan (Inutest, Laevosan, Linz, Austria) and 2% para-aminohippuric acid (PAH) (Merck Sharp & Dohme, West Point, PA) via the cannula in the jugular vein during the experimental period. Forty-five minutes was allowed for the preparation to reach a steady state. Timed urine collections were obtained, with blood (0.6 ml) collected between clearance periods. For maintenance of hematocrit, red blood cells from each blood sample were reconstituted to the same volume with 0.9% NaCl and reinjected through the arterial cannula. At the end of each experiment, kidneys were excised, blotted, and weighed. Urine volume was determined gravimetrically. Polyfructosan and PAH concentrations were determined by modified anthrone and colorimetric methods, respectively [85]. Glomerular filtration rate (GFR) and renal plasma flow (RPF) were determined from the clearance of polyfructosan and PAH, respectively. Renal blood flow (RBF) was calculated from RPF and hematocrit. Clearance data were normalized to kidney weight.

Morphological and histological investigation of the heart and kidney.

Rats were anesthetized with pentobarbital (50 mg/kg body weight) and hearts and kidneys were removed, cleaned, washed in saline, blotted and weighed. Slides of the kidney and heart were preserved in 4% buffered formaldehyde solution and embedded in paraffin. Five μm sections were cut with a microtome, mounted on glass slides and stained with hematoxylin-eosin, then analyzed microscopically and morphometrically. Histological sections of rat heart muscle were analyzed from all experimental groups. Cardiac myocyte diameters were measured in two perpendicular directions using an ocular micrometer with an engraved measuring scale [81]. The ocular micrometer was calibrated against a stage micrometer, and conversion factors were calculated for low (x 4 objective) and high (x 45 objective) magnifications. Cardiac myocytes were judged to be cut in cross section when the shorter measurement was not more than 2 μm wider than the longer measurement. The average of the two measurements was then recorded as the cross-sectional diameter of the measured myocyte. The mean diameter of 200 cardiomyocytes in each group was measured with a calibrated eyepiece at a magnification of x 450. All sections were evaluated in a blind study in which knowledge of the group to which the measurements belonged was revealed only after the data were tabulated.

Statistical analysis.

Data were analyzed using standard statistical methods. Repeated blood pressure measurements at each time point were taken for comparison between control and experimental groups. The blood pressure data were analyzed with the use of ANOVA and Fisher's protected least significant differences. Group data are expressed as mean ± SEM. Values were considered significantly different at a value of $P<0.05$.

Kallikrein gene delivery reduces blood pressure in hypertensive Dahl-SS rats on a high salt diet.

Dahl-SS rats (4 weeks old) were fed a high salt (4% NaCl) diet or normal control diet (rat chow, 0.4% NaCl) for 4 weeks. The blood pressure of Dahl-SS

rats on a 4% NaCl diet increased with the change in dietary salt intake and the differences between low and high salt groups reached over 50 mmHg prior to kallikrein gene delivery. Rats (8 weeks old) on a high salt diet were then divided into two groups and injected via the tail vein with either a viral vector containing the human tissue kallikrein gene (Ad.CMV-cHK) or a control vector containing the bacterial LacZ gene (Ad.CMV-LacZ). The blood pressure of Dahl-SS rats fed a high salt diet increased markedly as compared to rats on a low salt diet (0.4% NaCl). Delivery of the human tissue kallikrein gene resulted in a significant reduction of blood pressure in salt-induced hypertensive Dahl-SS rats at 4 and 11 days post injection. The difference in blood pressure between the control group and the group receiving kallikrein gene delivery persisted for more than 2 weeks post injection.

Expression of human tissue kallikrein after gene delivery.

Expression of the human tissue kallikrein mRNA in Dahl-SS rats after gene delivery was detected by RT-PCR followed by Southern blot analysis using three oligonucleotides specific for human tissue kallikrein. Total RNAs were prepared from tissues of rats 12 days after gene delivery. Human kallikrein mRNA can be detected in the kidney, heart, aorta and liver. The RT-PCR products from rats receiving the Ad.CMV-LacZ gene did not hybridize to the human tissue kallikrein gene probe. Similar levels of β actin mRNA were detected in tissues of both experimental and control groups, verifying the quality of RNA in these samples. These results indicate that Southern blot analysis is specific for human tissue kallikrein and that endogenous rat tissue kallikrein family members do not interfere with the assay.

Following intravenous injection of Ad.CMV-cHK adenovirus, human tissue kallikrein levels in rat sera and urine, collected at different time periods, were measured by ELISA. The highest level of immunoreactive human tissue kallikrein in rat serum was 254.1 ± 0.9 ng/ml on the third day after gene delivery. Also, immunoreactive human tissue kallikrein was measured in the urine of Dahl-SS rats receiving Ad.CMV-cHK (16.3 ± 3.6 μg/100 g body weight/day) but

not in the urine of control rats receiving Ad.CMV-LacZ. Linear displacement curves for immunoreactive kallikrein in rat sera and urine were parallel with the standard curve of human tissue kallikrein, indicating their immunological identity.

Increased urinary excretion, kinin, NOx, cAMP, cGMP and human tissue kallikrein levels in rats receiving kallikrein gene delivery.

Urinary excretion, kinin, NOx, cAMP and cGMP levels in Dahl-SS rats were measured at 7 days post gene delivery. Urine volume significantly increased in rats receiving kallikrein gene delivery as compared to control rats (13.7 ± 0.7 versus 9.2 ± 1.5 ml/100 g body weight per day, mean ± SEM; n=6, $P<0.05$). Urinary kinin levels increased by 3-fold after kallikrein gene delivery as compared to control rats receiving Ad.CMV-LacZ (35.4 ± 9.0 vs. 12.0 ± 4.4 ng/100 g body weight/day, n=5, $P<0.01$). Urinary cAMP content increased significantly after kallikrein gene delivery as compared to control rats receiving Ad.CMV-LacZ (18.5 ± 1.0 vs. 15.1 ± 0.9 nmol/100 g body weight/day, n=5, $P<0.05$). Urinary cGMP levels increased by 1.5-fold after kallikrein gene delivery as compared to control rats receiving Ad.CMV-LacZ (17.6 ± 1.9 vs. 11.4 ± 2.0 nmol/100 g body weight/day, n=5, $P<0.01$). Urinary NOx content increased significantly after kallikrein gene delivery as compared to control rats receiving the LacZ gene (0.83 ± 0.27 vs. 0.13 ± 0.07 μmole/100 g body weight/day, n=4, $P<0.05$).

Morphological changes in the heart after gene delivery.

The left ventricular weight is significantly increased in Dahl-SS rats on a high salt diet (4% NaCl) and injected with Ad.CMV-LacZ, as compared to control rats given a normal salt diet (0.4% NaCl) (0.44 ± 0.03 versus 0.27 ± 0.01 g per 100 g body weight, mean ± SEM; n=6, $P<0.05$). The left ventricular weight is significantly decreased in Dahl-SS rats given 4% NaCl in the diet, but injected with the tissue kallikrein gene (Ad.CMV-cHK) as compared to rats injected with Ad.CMV-LacZ (0.38 ± 0.04 versus 0.44 ± 0.03 g per 100 g body weight, mean ± SEM; n=6, $P<0.05$). The high salt loading resulted in an enlarged average diameter of cardiomyocytes in Dahl-SS rats with Ad.CMV-LacZ injection. The

average diameter of cardiomyocytes in the group receiving kallikrein gene transfer is significantly less than that of the Ad.CMV-LacZ group (16.8 ± 0.2 versus 20.7 ± 0.2 μm, mean ± SEM; n=200, P<0.01). Cardiac myocytes of animals on a normal salt diet (0.4% NaCl) appeared normal and uniform in diameter (13.6 ± 0.3 μm, mean ± SEM; n=200) and were used as the baseline of comparison for the high salt and gene-injected animals. Diffuse interstitial proliferation was occasionally found in the LacZ group but not in the group receiving kallikrein gene delivery. These results indicate that salt-induced cardiac hypertrophy can be at least partially reversed by kallikrein gene delivery in Dahl-SS rats.

Effects of kallikrein gene delivery on renal function.

Glomerular filtration rate in Dahl-SS rats fed a high salt diet and injected with control adenovirus Ad.CMV-LacZ decreased by 35% compared to control rats on a normal salt diet (0.31 ± 0.13 vs. 0.91 ± 0.08 ml/min/g kidney weight, mean ± SEM, n=4, P<0.05). After kallikrein gene delivery, glomerular filtration rate in Ad.CMV-cHK-injected rats increased 2.1-fold as compared to those rats injected with Ad.CMV-LacZ (0.66 ± 0.07 vs. 0.31 ± 0.13 ml/min/g kidney weight, mean ± SEM, n=4, P<0.05). Similarly, renal blood flow in Dahl-SS rats fed a high salt diet and injected with control adenovirus Ad.CMV-LacZ was reduced 56% compared to control rats on a normal salt diet (6.3 ± 1.9 vs. 11.8 ± 2.8 ml/min/g kidney weight, mean ± SEM, n= 4, P<0.05) while kallikrein gene delivery caused a 1.9-fold increase as compared to those rats injected with Ad.CMV-LacZ (12.0 ± 2.0 vs. 6.3 ± 1.9 ml/min/g kidney weight, mean ± SEM, n= 4). These results suggest that salt-induced renal damage leads to impairment of glomerular filtration and renal blood flow, and that kallikrein gene delivery causes reversal of renal damage.

Morphological changes in the kidney after gene delivery.

Histological sections of the renal cortex and medulla, stained with hematoxylin and eosin, showed some reversal of salt-induced renal injury in Dahl-SS rats after kallikrein gene delivery. The cortex and medulla of control Dahl-SS rats fed a normal salt diet generally appeared normal, although small

casts were occasionally seen in medullary tubules. High salt loading for 4 weeks resulted in significant renal injury in both the cortex and the medulla. In the cortex of animals treated with either high salt alone (4 weeks) or high salt (6 weeks) plus Ad.CMV-LacZ for 2 weeks, damage was marked. This included decreased cell height and loss of brush borders in proximal tubules, thickening of glomerular basement membranes with apparent glomerular sclerosis, and focal accumulation of inflammatory cells. Sites of focal (micro) hemorrhage were observed in Dahl-SS rats fed a high salt diet with and without Ad.CMV-LacZ for 2 weeks. Thickening of the arterial muscular layer (media) was obvious in both arcuate and interlobular arteries in these animals. In Dahl-SS fed a high salt diet and receiving kallikrein gene delivery (Ad.CMV-cHK), proximal tubules and glomeruli exhibited much less damage. No examples of hemorrhage were observed and inflammatory cell infiltration appeared decreased. The arterial media was, in most cases, intermediate in thickness between low-salt animals and high-salt animals receiving Ad.CMV-LacZ for 2 weeks. In addition, it is intriguing that mitoses were often seen in proximal tubule cells, and occasionally in collecting duct cells in animals receiving kallikrein gene delivery (Ad.CMV-cHK).

A protective effect of salt-induced glomerular sclerosis in Dahl-SS rats after kallikrein gene delivery was demonstrated. Of 75 glomeruli counted in control rats on a low salt diet, only $1.0 \pm 1.3\%$ exhibited sclerosis, compared with $25.8 \pm 4.5\%$ of 90 glomeruli counted in LacZ animals fed a high salt diet for 6 weeks ($P<0.01$). Of 70 glomeruli counted in Dahl-SS rats receiving kallikrein gene therapy and a high salt diet, $12.1 \pm 3.8\%$ exhibited sclerotic changes which is a 50% reduction of glomerular damage, compared to the LacZ group ($P<0.05$).

In the medulla, salt loading resulted in the development of large colloidal casts in renal tubules. These were also present in animals receiving the control LacZ gene (Ad.CMV-LacZ), but greatly reduced in rats receiving kallikrein gene delivery (Ad.CMV-cHK). These results indicate that adenovirus-mediated

kallikrein gene delivery improved salt-induced renal dysfunction and partially reversed morphological evidence of injury in Dahl-SS rats.

Example X. Atrial Natriuretic Peptide Gene Delivery Reduces Stroke-Induced Mortality Rate in Dahl Salt-Sensitive Rats.

Materials.

Dahl salt-sensitive rats (Dahl-SS, male, 4 weeks old) (Sprague-Dawley Harlan, Indianapolis, IN) were used in this study. Rats were divided into two groups. The first group was fed a standard rat chow (0.4% NaCl)) (Harlan Teklad, Madison, WI). The other group was fed a high salt diet (4% NaCl) (Harlan Teklad, Madison, WI). All rats had free access to water. Throughout the study period, all animals were housed in a room that was kept at constant temperature (25 ± 1°C) and humidity (60 ± 5%) and was lighted automatically from 8:00 am to 8:00 pm. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD).

Preparation of replication-deficient adenovirus vector Ad.RSV-cANP.

Plasmid RSV-cANP was constructed as previous described [86], in which the expression of human atrial natriuretic peptide cDNA (456 bp) was under the control of the Rous sarcoma virus long terminal repeat (RSV-LTR) and was followed by a SV40 poly A signal sequence. The transcription unit of RSV-cANP-poly A (1618 bp), including the RSV-LTR, the human atrial natriuretic peptide cDNA, and a Simian virus 40 poly A signal sequence, was released from the RSV-cANP plasmid by Sal I digestion. Plasmid pAd.RSV-cANP was constructed by inserting the released fragment into the adenovirus shuttle vector pAdLink.1 (adenoviral capacity, 8 kb) at a Sal I site. The pAd.RSV-cANP plasmid DNA was purified using a Qiagen plasmid DNA kit (Qiagen, Chatsworth, CA) [87]. The purified DNA was sent to the Institute for

Human Gene Therapy, Wistar Institute, Philadelphia, for generation of adenovirus Ad.RSV-cANP harboring the RSV-cANP-poly A transcription unit. Adenovirus harboring the LacZ gene under the control of the Rous sarcoma virus promoter (Ad.RSV-LacZ) was purchased from the Institute for Human Gene Therapy, Philadelphia.

Intravenous Delivery of Adenoviral Vectors Ad.RSV-cANP and Ad.RSV-LacZ.

Twenty-seven Dahl-SS rats fed a high salt diet containing 4% NaCl for 4 weeks were randomly divided into three groups and were intravenously (IV) injected with either Ad.RSV-cANP (n=13) or Ad.RSV-LacZ (n=7) at a dosage of 2.4×10^{10} pfu (plaque formation unit) per rat through the tail vein. Seven Dahl-SS rats on a 4% NaCl diet did not receive any adenovirus injection.

Blood pressure measurement.

The systolic blood pressure of rats was measured with a manometer-tachometer (Nastume KN-210; Nastume Seisakusho Co. Ltd., Tokyo, Japan) using the tail-cuff method [86]. Unanesthetized rats were introduced into a plastic holder mounted on a thermostatically controlled warm plate, which was maintained at 33-35°C during the measurement. An average of ten readings was taken for each animal.

Tissue preparation.

At 3 days after gene delivery, rats from each group were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight and perfused with normal saline (0.9% NaCl) by cardiac puncture. Tissues were homogenized in normal saline with a Polytron (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 600 x g for 10 minutes. The supernatant was incubated in 0.5% sodium deoxycholate and then centrifuged at 10,000 x g for 30 minutes. Total protein in the supernatant was determined by Lowry's method [88]. At 4 weeks after ANP gene delivery, all survivals were sacrificed. Brains and thoracic aortas were immediately removed and processed.

Radioimmunoassay (RIA) for human ANP.

The level of human atrial natriuretic peptide in each tissue extract was determined by a RIA specific for human ANP. Ten micrograms of human synthetic ANP (Ser 99-Tyr 126) (Sigma Chemical Co., St. Louis, MO) was labeled with 1 mCi of ^{125}-Iodine which was iodinated with iodogen for 10 min at room temperature. The iodinated ANP in 250 mmol/L sodium phosphate buffer, pH 7.0, was separated on a reverse-phase C-18 HPLC column in an acetonitrile gradient. ^{125}I-ANP labeled tracer which was eluted from the column at 19-20 min post-injection was identified by antibody titration. Serial dilutions of standard ANP (10 pg-1280 pg) or tissue extracts (100 µL) were incubated with goat-anti-human ANP antiserum (1: 1500 dilution, Sigma Chemical Co., St. Louis, MO) in a solution containing 0.01 mol/L PBS, pH 7.4, 0.3% BSA, 0.1% Triton X100, 0.1 mmol/L EDTA and 0.1% sodium azide, and ^{125}I-ANP tracer (10,000 cpm in 100 µL) in a total volume of 400 µL for 18-24 hours at 4°C. The reaction was stopped by adding 800 µL of 25% polyethylene glycol (PEG) in PBS containing 0.1% sodium azide and 400 µL of 1% bovine gamma-globulin in PBS containing 0.1% sodium azide. The radioactivity of the precipitate was determined in a gamma counter.

Monitoring of stroke development.

The rats were monitored daily for the occurrence of stroke. The symptoms associated with stroke development have been previously described for SHRSP [89,90]. Initially, SHRSP develop convulsive repetitive forearm movement followed by inappropriate posture during which rats sit with legs hyperextended in a "kangaroo-type" posture. In this study, the symptom associated with Dahl-SS rats was often associated with lethargy and poor grooming. There is no typically fixed period between the onset of the first behavioral symptom of stroke and death. Some animals died abruptly after the first behavioral symptom of stroke. While others were sacrificed at a point when death was likely to occur within a day.

Confirmation of infarction area of stroke brain.

Serial coronal brain sections (2 mm in thickness) were prepared and were stained with 2, 3, 5-triphenyl-tetrazolium chloride (TTC) [91] (Sigma Chemical Co.). Brain slices were immersed in normal saline containing 2% TTC at 37°C for 30 minutes. TTC, a colorless salt, is reduced to form an insoluble red formazan product in the presence of a functioning mitochondrial electron transport chain. Thus, the infarcted region lacks staining and appears white, whereas the normal, noninfarcted tissue appears red.

Morphological and histological investigations.

Sections of the brain and thoracic aorta were preserved in 4% buffered formaldehyde solution and paraffin embedded. Five μm-thick sections were cut and stained with hematoxylin-eosin (HE) and analyzed microscopically and morphometrically. Measurements of the thickness of aortic wall were performed as described. Ten measurements taken from different positions of each aorta were averaged. All sections were evaluated by independent personnel with no prior knowledge of the group from which rats were obtained.

Statistical analysis.

Repeated blood pressure measurements at each time point were taken after gene delivery for comparison between control and experimental groups, and data were analyzed with the use of either unpaired Student's t-test or ANOVA and Fisher's protected least significant differences. Group data were expressed as mean ± SEM. Survival curves were constructed using Kaplan-Mier analysis. Statistical significance of these data was measured by analysis of variance and χ^2, using a SAS software package. Values of blood pressures and other parameters were considered significantly different at a value of $P<.05$.

Blood pressure reduction after intravenous injection of the human ANP gene.

Dahl-SS rats (4 weeks old) were fed a high salt (4% NaCl) diet or normal rat chow (0.4% NaCl) as controls for 4 weeks until blood pressure differences between these two groups reached over 45 mmHg. Dahl-SS rats on the high salt

diet were divided into three groups and two groups were intravenously injected with either adenovirus Ad.RSV-cANP carrying the ANP gene or control virus Ad.RSV-LacZ containing the LacZ gene through the tail vein. One group was not given any adenovirus injection. Blood pressures of these rats were monitored weekly for 3 weeks post gene delivery. Delivery of the human ANP gene caused a significant reduction of blood pressure at 1 week after injection and the effect lasted for more than 3 weeks. A maximal blood pressure reduction of 28 mmHg was observed 14 days after ANP gene delivery as compared to that of rats injected with control virus Ad.RSV-LacZ (219.9 ± 5.9 vs. 247.9 ± 3.1 mmHg, mean ± SEM, n=6, P<.01). In contrast, blood pressures of control rats on a normal salt diet (0.4% NaCl) remained around 135-155 mm Hg throughout the experimental period.

Expression of human ANP after gene delivery.

Expression levels of human ANP in Dahl-SS rats were analyzed by a RIA specific for human ANP. Immunoreactive human ANP was detected in the heart (35.9 ± 3.4 ng/mg protein, n=3), lung (6.5 ± 0.3 ng/mg protein, n=3), kidney (15.3 ± 0.9 ng/mg protein, n=3), and brain (104.5 (18.6 pg/mg protein, n=3) 3 days after intravenous injection of the human ANP gene. Linear displacement curves for immunoreactive ANP in the heart, lung, brain and kidney of Dahl-SS rats were parallel with the standard curve of human ANP, indicating their immunological identity. Serial dilutions of the heart, lung and kidney extracts from control rats injected with Ad.RSV-LacZ showed a lack of parallelism with the human ANP standard curve. These results indicate that goat anti-human ANP antibody has some cross-reactivity with rat ANP, however, human and rat ANP are not immunologically identical and are distinguishable in the RIA.

Mortality rate of Dahl-SS rats with stroke.

Dahl-SS rats began to show symptoms of stroke including lethargy, poor grooming, convulsive repetitive forearm movement or semiplegia at five and a half weeks after high salt loading. Some animals died rapidly after the first behavioral symptom of stroke. Kaplan-Meier survival plots for Dahl-SS rats after

ANP gene delivery were analyzed. At 3 weeks after ANP gene delivery (51 days after high salt loading), the survival rates were 100% in control (0.4% NaCl diet), 83% in the Ad.RSV-cANP group (4% NaCl), and 46% in high salt loading (4% NaCl diet alone and Ad.RSV-LacZ) groups. At 4 weeks after adenovirus injection (58 days after high salt loading), 70% of Dahl-SS rats fed a high salt with or without LacZ adenovirus injection died from stroke. Cumulatively, 50% of Dahl-SS rats in the Ad.RSV-cANP group survived. The Kaplan-Meier plots were analyzed statistically by χ^2, generating a P value of $<.05$. Pathological changes in coronal brain sections including hemorrhage, edema and focal infarction were observed in Dahl-SS rats with stroke at 4 weeks after gene delivery. Focal infarction regions in the brain from individuals of the high salt group were stained white with 2% TTC. After ANP gene delivery, brain sections of Dahl-SS rats appeared reddish and relatively normal.

Human ANP gene delivery reduced salt-induced aortic thickening in Dahl-SS rats.

The thickness of aortic wall was significantly reduced in the Ad.RSV-cANP group at 4 weeks after gene delivery as compared to that of the Ad.RSV-LacZ group (153.5 ± 2.2 vs. 202.8 ± 18.1 μm, mean ± SEM, n=5, $P<.05$), while the aortic wall of Dahl-SS rats on a 0.4% NaCl diet was 136.7 ± 3.5 μm in thickness. These results indicate that human ANP gene delivery can attenuate, at least in part, salt-induced aortic hypertrophy in hypertensive Dahl-SS rats.

Although the present invention has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

REFERENCES

1. Clements JA. The glandular kallikrein family of enzymes: tissue-specific expression and hormonal regulation. Endocrine Rev. 1989;10:393-419.

2. Bhoola KD, CD Figueroa, K Worthy. Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol. Rev. 1992;44:1-8.

3. Margolius HS, R Geller, W de Jong, JJ Pisano, A Sjoerdsma. Altered urinary kallikrein excretion in human hypertension. Lancet. 1971;ii:1063-1065.

4. Margolius HS, D Horwitz, JJ Pisano, HR Keiser. Urinary kallikrein excretion in hypertensive man. Relationships to sodium intake and sodium-retaining steroids. Circ Res. 1974;35:820-825.

5. Zinner SH, HS Margolius, B Rosner, EH Kass. Stability of blood pressure rank and urinary kallikrein concentration in childhood: An eight year follow-up. Circulation. 1978:58:908-915.

6. Berry TD, SJ Hasstedt, SC Hunt, LL Wu, JB Smith, KO Ash, H Kuida, RR Williams. A gene for high urinary kallikrein may protect against hypertension in Utah kindreds. Hypertension. 1989;13:3-8.

7. Ader JL, DM Pollock, MI Butterfield WJ Arendshorst. Abnormalities in kallikrein excretion in spontaneously hypertensive rats. Am J. Physiol. 1985;248:F396-F403.

8. Favaro S, B Baggio, A Antonello, A Zen, G Cannella, S Todesco, A Borsatti. Renal kallikrein content of spontaneously hypertensive rats. Clin Sci Mol Med. 1975;49:69-71.

9. Margolius HS, R Geller, W De Jong, JJ Pisano, A Sjoerdsma. Altered urinary kallikrein excretion in rats with hypertension. Circ Res. 1972;30:358-362.

10. Geller RG, HS Margolius, JJ Pisano, HR Keiser. Urinary kallikrein excretion in spontaneously hypertensive rats. Circ Res. 1975;36(Suppl. 1):103-106.

11. Bouhnik J, Richoux J P, Huang H, Savoie F, Baussant T, Alhenc-Gelas F, Corvol P. Hypertension in Dahl salt-sensitive rats: biochemical and immunohistochemical studies. Clin Sci. 1992;83:13-22.

12. de Bold AJ, Borenstein HB, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.* 1981;28:89-94

13. de Bold AJ. Atrial natriuretic factor: a hormone produced by the heart. *Science.* 1985;230:767-770

14. Needleman P, Adams SP, Cole BR, Currie MG, Geller DM, Michener ML, Saper CB, Schwartz D, Standaert DG. Atriopeptins as cardiac hormones. *Hypertension.* 1985;7:469-482

15. Laragh JH. Atrial natriuretic hormone, the renin-aldosterone axis, and blood pressure-electrolyte homeostasis. *N Engl J Med.* 1985;313: 1330-1340

16. Cantin M, Genest J. The heart and the atrial natriuretic factor. *Endocr Rev.* 1985;6:107-127

17. Needleman P, Greenwald JE. Atriopeptin: a cardiac hormone intimately involved in fluid, electrolyte, and blood-pressure homeostasis. *N Engl J Med.* 1986;314:828-834

18. Sonnenberg H. Renal effects of atrial natriuretic factor. *ISI Atlas of Science: Pharmacology.* 1988;171-174

19. Trippodo NC. An update on the physiology of atrial natriuretic factor. *Hypertension.* 1987;10(suppl I):I-122-I-127

20. Brenner BM, Ballermann BJ, Gunning ME, Zeidel ML. Diverse biological actions of atrial natriuretic peptide. *Physiol Rev.* 1990;70:665-699

21. Atlas S, Maack T. Atrial natriuretic factor. In: Windhager EE, ed. *Handbook of physiology. Renal Physiology* New York, NY: Oxford University Press 1992;1577-1673

22. Janssen WM , de Zeeuw D, van der Hem GK, de Jong PE. Antihypertensive effect of a 5-day infusion of atrial natriuretic factor in humans. *Hypertension.* 1989;13:640-646

23. Granger JP, Opgenorth TJ, Salazar J, Romero JC, Burnett JC Jr. Long-term hypotensive and renal effects of atrial natriuretic peptide. *Hypertension.* 1986;8(suppl II):II-112-II-116

24. Spokas EG, Suleymanov OD, Bittner SE, Campion JG, Gorczynski RJ, Lenaers A, Walsh GM. Cardiovascular effects of chronic high-dose atriopeptin III infusion in normotensive rats. *Toxicol Appl Pharmacol.* 1987;91:305-314

25. Pares DG, Coghlan JP, McDouggali JG, Scoggins BA. Long-term hemodynamic actions of atrial natriuretic factor (99-126) in conscious sheep. *Am J Physiol.* 1988;254:H811-H815

26. Harrison-Bernard LM, Vari RC, Holleman WH, Trippodo NC, Barbee RW. Chronic vs. acute hemodynamic effects of atrial natriuretic factor in conscious rats. *Am J Physiol.* 1991;260:R247-R254

27. Garcia R, Thibault G, Gutkowska J, Horky K, Hamet P, Cantin M, Genest J. Chronic infusion of low doses of atrial natriuretic factor (ANF Arg 101-Tyr 126) reduces blood pressure in conscious SHR without apparent changes in sodium excretion. *Proc Soc Exp Biol Med.* 1985;179:396-401

28. De Mey JG , Cuthbert C, von Szendroi KG, van Malder G, Roba J. Smooth muscle relaxing, acute and long-term blood pressure lowering effect of atriopeptins: structure-activity relationship. *J Pharmacol Exp Ther.* 1987;240:937-943

29. Simon WM , Krege JH , Oliver PM , Hagaman JR , Hodgin JB , Pang SC , Flynn TG , Smithies O. Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science.* 1995;267:679-681.

30. Sambrook et al. 1989. Molecular Cloning: A Laboratory Manual, 2d Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

31. Pastan et al. "A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells." *Proc. Nat. Acad. Sci.* 85:4486 (1988)

32. Miller et al. "Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production." *Mol. Cell Biol.* 6:2895 (1986)).

33. Mitani et al. "Transduction of human bone marrow by adenoviral vector." *Human Gene Therapy* 5:941-948 (1994)).

34. Goodman et al. "Recombinant adeno-associated virus-mediated gene transfer into hematopoietic progenitor cells." *Blood* 84:1492-1500 (1994))

35. Naidini et al. "*In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector." *Science* 272:263-267 (1996))

36. Agrawal et al. "Cell-cycle kinetics and VSV-G pseudotyped retrovirus mediated gene transfer in blood-derived CD34$^+$ cells." *Exp. Hematol.* 24:738-747 (1996)).

37. Schwarzenberger et al. "Targeted gene transfer to human hematopoietic progenitor cell lines through the *c-kit* receptor." *Blood* 87:472-478 (1996)).

38. Fields. *et al.* (1990) Virology, Raven Press, New York).

39. Martin, E.W. (ed.) Remington's Pharmaceutical Sciences, latest edition. Mack Publishing Co., Easton, PA.

40. Chao J, Jin L, Chen LM, Chen VC, Chao L. Systemic and portal vein delivery of human kallikrein gene reduces blood pressure in hypertensive rats. Human Gene Therapy 1996; 7: 901-911.

41. Wang J, Xiong W , Yang Z , Davis T , Dewey MJ , Chao J , Chao L. Human tissue kallikrein induces hypotension in transgenic mice. Hypertension. 1994;23:236-243.

42. Shimamoto K, Tanaka S, Nakao T, Ando T, Nakahashi Y, Sakuma M, Miyahara M: Measurement of urinary kallikrein activity by kinin radioimmunoassay. *Jpn Circ J* 43:147-152, 1979

43. Brooker G, Harper J F, Terasaki W L, Moylan R D. Radioimmunoassay of cyclic AMP and cyclic GMP. Adv Cyclic Nucleotide Res. 1979;10:1-33.

44. Harper J F, Brooker G. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O acetylation by acetic anhydride in aqueous solution. J. Cyclic Nucleotide Res. 1975;1:207-218.

45. Gettys T W, Okonogi K, Tarry W C, Johnston J, Horton C, Taylor I L. Examination of relative rates of cAMP synthesis and degradation in crude membranes of adipocytes treated with hormones. Second Messengers and Phosphoproteins. 1990;13:37-50.

46. Gettys T W, Ramkumar V, Uhing R J, Seger L, Taylor I L. Alterations in mRNA levels, expression, and function of GTP-binding regulatory proteins in adipocytes from obese mice (C57BL/6J-ob/ob). J Biol Chem. 1991;266:15949-15959.

47. Lin KF, Chao J, Chao L. Human atrial natriuretic peptide gene delivery reduces blood pressure in hypertensive rats. *Hypertension.* 1995;26:847-853.

48. Lin KF, L Chao, J Chao. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. *Hypertension.* 1997.

49. Wayne RF, Ayad AJ, Ronald KM, David WP. Role of kinins in the renal response to enalaprilat in normotensive and hypertensive rats. *Hypertension.* 1996;27:235-244.

50. Fuhr J, Kaczmarczk J, Kruttgen CD. Eine einfache colorimetrische methode zur inulin-bestimmung fur nieren-clearance-untersuchungen bei stoffwecksel-gesunden und diabetiken. *Klin Wochenschr.* 1955;33:729-730

51. Smith HW, Finkelstein N, Aliminosa L, Crawford B, Grabor M, Renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. *J Clin Invest.* 1945;24:388-404

52. Wang C, Chao L, Chao J: Direct gene delivery of human tissue kallikrein reduces blood pressure in spontaneously hypertensive rats. *J Clin Invest* 95:1710-1716, 1995

53. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH: Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ Res* 74:349-353, 1994.

54. Fitzgibbon WR, Jaffa AA, Mayfield RK, Ploth DW: Role of kinins in the renal response to enalapril in normotensive and hypertensive rats. *Hypertension* 27:235-244, 1996

55. Ali BH: Gentamicin nephrotoxicity in humans and animals: Some recent research. *Gen Pharmacol* 26:1477-1487, 1995.

56. Kacew S, Bergeron MG: Pathogenic factors in aminoglycoside-induced nephrotoxicity. *Toxicol Lett* 51:241-259, 1990

57. Collier VU, Lietman PS, Mitch WE: Evidence for luminal uptake of gentamCicin in the perfused rat kidney. *J Pharmacol Exp Ther* 210:247-251, 1979

58. Shimamoto et al., 1978. *J. Lab. Clin. Med.* 103:731-738.

59. Crystal, R.G. 1997. Phase I study of direct administration of a replication deficient adenovirus vector containing *E. coli* cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. *Human Gene Therapy* **8**:985-1001.

60. Alvarez, R.D. and D.T. Curiel. 1997. A phase I study of recombinant adenovirus vector-mediated delivery of an anti-erbB-2 single chain (sFv) antibody gene from previously treated ovarian and extraovarian cancer patients.

61. Lin KF, Chao J, Chao L. Human atrial natriuretic peptide gene delivery reduces blood pressure in hypertensive rats. Hypertension 1995; 26: 847-853.

62. Bauer JD. Carbohydrates and nitrogen compounds. In Bauer JD, ed. Clinical Laboratory Methods (9th ed). The C.V. Mosby Company, St. Louis: 1982: 472-505.

63. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.

64. Lin KF, Chao L, Chao J. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. Hypertension 1997; 30[part 1]: 307-313.

65. Fitzgibbon WR, Jaffa AA, Mayfield RK, Ploth DW. Role of kinins in the renal response to enalapril in normotensive and hypertensive rats. Hypertension 1996; 27: 235-244.

67. Wang C, Chao L, Chao J. Direct gene delivery of human tissue kallikrein reduces blood pressure in spontaneously hypertensive rats. J Clin Invest. 1995;95:1710-1716.

68. Lowry OH, Roserbrough NJ, Farr AL. Protein measurement with the folin phenol reagent. J Biol Chem. 1951;193: 265-275.

69. Johnson DA, Salvesen G, Brown MA, Barrett AJ. Rapid isolation of human kininogens. Thrombosis Res 1987; 48: 187-193.

70. Shimamoto K, Ando T, Nakao T, Tanaka S, Sakuma MA, Miyahara M. A sensitive radioimmunoassay method for urinary kinins in man. J Lab Clin Med. 1987;91:721-728.

71. Lin KF, Chao L, Chao J. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. Hypertension. 1997;30:307-313.

72. Smeda JS. Hemorrhagic stroke development in spontaneously hypertensive rats fed a North American, Japanese-style diet. Stroke. 1989;20:1212-1218.

73. Smeda JS. Cerebral vascular changes associated with hemorrhagic stroke in hypertension. Can J Physio. 1992;70: 552-564.

74. Chao J, Zhang J, Lin KF, Chao L. Adenovirus-mediated kallikrein gene delivery attenuates hypertension, cardiac hypertension and renal injury in Dahl-salt sensitive rats. Human Gene Ther. 1998;9:21-31.

75. Becker TC, Noel RF, Schmid S, Hearing P. Methods Cell Biol. 43; 161-189, 1994.

76. Hall KL, Harding JW, Hosick HL. Isolation and characterization of clonal vascular smooth muscle cell lines from spontaneously hypertensive and normotensive rat aortas. In Vitro Cell. Devel. Biol. 1991;27A:791-798.

77. Gordon D, Mohai LG, Schwartz SM. Induction of polyploidy in cultures of neonatal rat aortic smooth muscle cells. Circ. Res. 1986;59:633-644.

78. Johnson DA, Salvesen G, Brown MA, Barrett AJ. Rapid isolation of human kininogens. Thrombosis Res. 1987;48:187-193.

79. Shimamoto K, Tanaka S, Nakao T, Ando T, Nakahashi Y, Sakuma M, Miyahara M. Measurement of urinary kallikrein activity by kinin radioimmunoassay. Jpn. Circ. J. 1979;43: 147-152.

80. Lin KF, Chao L, Chao J. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. Hypertension. 1997;30:307-313.

81. Chao J, Zhang JJ, Lin KF, Chao L: Human kallikrein gene delivery attenuates hypertension, cardiac hypertrophy and renal injury in Dahl salt-sensitive
rats. Hum Gene Ther 9:21-31, 1998.

82. Wang C, Chao L, Chao J: Direct gene delivery of human tissue kallikrein reduces blood pressure in spontaneously hypertensive rats. J Clin Invest 95:1710-1716, 1995.

83. Lin KF, Chao L, Chao J: Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. Hypertension 30:307-313, 1997.

84. Brooker G, Harper JF, Terasaki WL, Moylan RD: Radioimmunoassay of cyclic AMP and cyclic GMP. Adv in Cycl Nucl Res 10:1-33, 1979.

85. Fitzgibbon WR, Jaffa AA, Mayfield RK, Ploth DW: Role of kinins in the renal response to enalapril in normotensive and hypertensive rats. Hypertension 27:235-244, 1996.

86. Lin K F, Chao J, Chao L. Human atrial natriuretic peptide gene delivery reduces blood pressure in hypertensive rats. Hypertension. 1995;26:847-853.

87. Lin K F, Chao J, Chao L. Atrial Natriuretic peptide gene delivery attenuates hypertension, cardiac hypertrophy and renal injury in salt-sensitive rats. Hum Gene Ther. 1998;9:1429-1438.

88. Lowry O H, Rosebrough N J, Farr A L, Randall R J. Protein measurement with the folin phenol reagent. J Bio Chem. 1951;193:265-275.

89 Smeda J S. Hemorrhagic stroke development in spontaneously hypertensive rats fed a North American, Japanese-style diet. Stroke. 1989;20:1212-1218.

90. Smeda J S. Cerebral vascular changes associated with hemorrhagic stroke in hypertension. Can J Physiol Pharmacol. 1992;70:552-564.

91. Bederson J B, Pitts L H. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke. 1986;17:1304-1308.

92. Margolius H.S. Tissue kallikrein and kinins: regulation and roles in hypertensive and diabetic diseases. Annu Rev Pharmacol Toxic. 1989;29:343-364.

Table 1. Physiological Analysis of DS Rats Injected with Ad.CMV-cHK and Ad.CMV-LacZ

Variables	Ad.CMV-LacZ	Ad.CMV-cHK
Systolic blood pressure (mmHg)	170.9 ± 0.3	162.8 ± 0.6 †
Heart rate (beats/min)	421 ± 14	467 ± 4
Body weight (grams)	219.2 ± 6.5	214.2 ± 2.3
Water intake (mL/100g body wt per day)	3.5 ± 0.9	11.7 ± 2.3 *
Urine volume (mL/100g body wt per day)	6.8 ± 0.9	12.9 ± 2.0 *
Urinary Na output (mmol/100g body wt per day)	0.27 ± 0.06	0.40 ± 0.02 *
Urinary K output (mmol/100g body wt per day)	0.49 ± 0.05	0.58 ± 0.05
Urinary creatinine (mg/100g body wt per day)	2.29 ± 0.03	2.9 ± 0.3
Urinary protein (mg/100g body wt per day)	20.0 ± 5.0	22.6 ± 1.7
Urinary kinin (nmol/100g body wt per day)	9.9 ± 2.2	50.7 ± 6.7 †
Urinary cGMP (nmol/100g body wt per day)	7.6 ± 1.0	10.7 ± 2.1 *

Young DS rats received either Ad.CMV-cHK or Ad.CMV-LacZ injection at the age of five and a half weeks old and physiological measurements of rats were performed nine days post gene delivery. Values for each group are reported as mean±SEM (n=6). Six rats from each group were measured for systolic pressure, heart rate, body weight, water intake, urine output, and urinary electrolytes. Statistical significance between the two groups was determined by the unpaired Student's t -test. A value of $P>0.05$ was interpreted as indicating an insignificant difference between the groups. † $P<0.01$. *$P<0.05$.

Table 2. Morphological Changes of DS Rats after Ad.CMV-cHK Gene Delivery

Variables	(1) Control (0.44%NaCl)	(2) Ad.CMV-LacZ (4% NaCl)	3) Ad.CMV-cHK (4% NaCl)
Whole cardiac weight (g/100g body wt)	0.36 ± 0.01 †	0.49 ± 0.04	0.46 ± 0.02
Left ventricular weight (g/100g body wt)	0.31 ± 0.06 *	0.38 ± 0.04	0.36 ± 0.02 **
Cardiomyocyte diameter (μm)	14.7 ± 0.6 *	19.1 ± 0.6	16.8 ± 0.3 **
Left renal mass (g/100g body wt)	0.45 ± 0.03 †	0.58 ± 0.06	0.55 ± 0.06
Right renal mass (g/100g body wt)	0.44 ± 0.02 †	0.59 ± 0.05	0.55 ± 0.05

Data were analyzed with ANOVA. Values for each group are reported as mean±SEM (n=6). *represents $P<0.05$, group (1) versus group (2) or (3); † represents $P<0.01$, group (1) versus group (2) or (3). **represents $P<0.05$, group (3) versus group (2).

Table 3. Physiological Analysis of DS Rats Injected with Ad.RSV-cANP and Ad.CMV-LacZ

Variables	Ad.CMV-LacZ	Ad.RSV-cANP
Systolic blood pressure (mmHg)	172.3 ± 1.6	155.3 ± 1.17 †
Heart rate (beats/min)	402 ± 8	377 ± 16
Body weight (grams)	166.2 ± 2.2	166.8 ± 3.6
Water intake (mL/100g body wt per day)	9.9 ± 2.7	12.0 ± 3.0
Urine volume (mL/100g body wt per day)	11.8 ± 1.7	16.7 ± 2.7 *
Urinary Na output (mmol/100g body wt per day)	0.44 ± 0.07	0.72 ± 0.06 *
Urinary protein (mg/100g body wt per day)	64.7 ± 2.8	88.0 ± 15.4
Urinary cGMP (nmol/100g body wt per day)	7.62 ± 1.02	36.43 ± 13.73 *

Young DS rats received either Ad.RSV-cANP or Ad.CMV-LacZ injection at the age of five and a half weeks old and physiological measurements of rats were performed eleven days post gene delivery. Values for each group are reported as mean±SEM (n=6). Six rats from each group were measured for systolic pressure, heart rate, body weight, water intake, urine output, and urinary electrolytes. Statistical significance between the two groups was determined by the unpaired Student's *t* -test. A value of $P>0.05$ was interpreted as indicating an insignificant difference between the groups. †$P<0.01$. * $P<0.05$.

Table 4. Morphological Changes of Dahl-SS Rats after Ad.RSV-cANP Gene Delivery

Variables	(1) Control (0.4%NaCl)	(2) Ad.CMV-LacZ (4% NaCl)	3) Ad.RSV-cANP (4% NaCl)
Whole cardiac weight (g/100g body wt)	0.35 ± 0.01 †	0.54 ± 0.04	0.43 ± 0.03
Left ventricular weight (g/100g body wt)	0.24 ± 0.01 †	0.41 ± 0.04	0.32 ± 0.003 **
Cardiomyocyte diameter (μm) (n=120)	13.27 ± 0.10 *	19.07 ± 0.16	15.22 ± 0.13 **
Average renal mass (g/100g body wt)	0.41 ± 0.01 †	0.61 ± 0.01	0.55 ± 0.03 **
Aortic weight (g/100g body wt)	0.03 ± 0.002 †	0.04 ± 0.004	0.03 ± 0.01

Data were analyzed with ANOVA. Values for each group are reported as mean ± SEM (n=6). * represents P<0.05, group (1) versus group (2) and (3); † represents P<0.01, group (1) versus group (2) and (3); ** represents P<0.05, group (3) versus group (2).

Table 5. Effects on Renal Function of Dahl-SS Rats after Ad.RSV-cANP Gene Delivery

Variables	(1) Control (0.4%NaCl)	(2) Ad.CMV-LacZ (4% NaCl)	3) Ad.RSV-cANP (4% NaCl)
Urine flow rate (μl/min/g kidney wt)	6.12 ± 2.75	5.61 ± 1.77	2.50 ± 0.65 **
Glomerular filtration rate (ml/min/g kidney wt)	1.19 ± 0.17 *†	0.17 ± 0.05	0.56 ± 0.06 **
PAH clearance (mg/min/g kidney wt)	1.93 ± 0.64 *	0.04 ± 0.02	1.78 ± 0.71 **
Renal blood flow (ml/min/g kidney wt)	7.90 ± 1.11 *	0.18 ± 0.07	6.95 ± 0.31 **
Hematocrit (%)	57 ± 3 *	44 ± 6	56 ± 2 **

Data were analyzed with ANOVA. Values for each group are reported as mean ± SEM (n=3 or 4). * represents $P<0.05$, group (1) versus group (2); † represents $P<0.05$, group (1) versus (3). ** represents $P<0.05$, group (3) versus group (2).

Table 6. Effects of Human Kallikrein Gene Delivery on Urine Parameters of Gentamycin Nephrotoxic Rats

	Groups		
Variables	Control	Gentamycin Ad.CMV-LacZ	Gentamycin Ad.CMV-cHK
Body Weight (g)	263 ± 4.0	251.4 ± 3.9	238.4 ± 2.5
Urine Volume (ml/day/100g BW)	6.0 ± 0.6	9.7 ± 1.5	10.9 ± 1.8
Water Intake (ml/day/100g BW)	4.2 ± 1.2	6.5 ± 1.4	11.4 ± 1.4
Human Tissue Kallikrein (μg/100g BW/day)		n.d.	3.2 ± 0.9
Urinary Kinin (ng/100g BW/day)	21.9 ± 4.6	26.5 ± 4.5	78.4 ± 24.1[a]
Urinary Nitric Oxide (μM/100g BW/day)	392.5 ± 125.6	593.1 ± 21.3	871.2 ± 92.8[a]

Gentamycin-induced nephrotoxicity in rats receiving adenoviral vectors Ad.CMV-cHK or Ad.CMV-LacZ via the tail vein on the first day of gentamycin administration. Urine collection was performed seven days after gene delivery. Values for each group are reported as mean ± SEM (n=3). Statistical significance among the three groups were determined by ANOVA.

[a] $P<0.05$ with respect to the gentamycin and Ad.CMV-LacZ group.

Table 7. Effects of Human Kallikrein Gene Delivery on Renal Function of Gentamycin Nephrotoxic Rats

	Groups			
Variables	Control	Gentamycin	Gentamycin Ad.CMV-LacZ	Gentamycin Ad.CMV-cHK
UF (ml/min/g KW)	5.7 ± 0.4	4.4 ± 0.3	5.0 ± 0.2	18.5 ± 0.4[a]
GFR (ml/min/g KW)	0.94 ± 0.06	0.73 ± 0.05	0.83 ± 0.04	3.07 ± 0.07[a]
RBF (ml/min/g KW)	11.1 ± 0.9	8.7 ± 0.8	9.2 ± 0.6	32.0 ± 2.1[a]
KW (g/100g BW)	0.74 ± 0.04	1.19 ± 0.14	1.05 ± 0.08	1.05 ± 0.05

Gentamycin-induced nephrotoxicity in rats receiving adenoviral vectors Ad.CMV-cHK or Ad.CMV-LacZ via the tail vein on the first day of gentamycin administration. Renal function study was performed and kidney weights were measured at ten days after gene delivery. UF, urine flow; GFR, glomerular filtration rate; RBF, renal blood flow; KW, kidney weight; BW, body weight. Values for each group are reported as mean ± SEM (n=4). Statistical significance among the four groups were determined by ANOVA.

[a] $P<0.01$ with respect to gentamycin and Ad.CMV-LacZ group

What is claimed is:

1. A method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating a nonhypertension-associated renal disorder.

2. The method of claim 1, wherein the nucleic acid is administered to the subject in a virus.

3. The method of claim 2, wherein the virus is selected from the group consisting of adenovirus, retrovirus and adeno-associated virus.

4. The method of claim 2, wherein the virus is adenovirus.

5. The method of claim 1, wherein the nucleic acid is administered to the subject in a liposome.

6. The method of claim 1, wherein the cell is a kidney cell.

7. The method of claim 1, wherein the cell is *in vivo*.

8. The method of claim 1, wherein the cell is *ex vivo*.

9. The method of claim 1, wherein the nonhypertension-associated renal disorder is selected from the group consisting of renal injury, nephrotoxicity, nonhypertension-associated renal disease; salt-induced renal damage, glomerulosclerotic lesions, tubular injury, drug-induced renal damage, chronic renal failure, acute renal failure, nephrotic syndrome and diabetic nephropathy.

10. A method of treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein under conditions whereby the nucleic acid encoding tissue kallikrein is expressed in a cell in the subject, thereby treating the nonhypertension-associated cardiac disorder.

11. The method of claim 10, wherein the nucleic acid is administered to the subject in a virus.

12. The method of claim 11, wherein the virus is selected from the group consisting of adenovirus, retrovirus and adeno associated virus.

13. The method of claim 11, wherein the virus is adenovirus.

14. The method of claim 10, wherein the nucleic acid is administered to the subject in a liposome.

15. The method of claim 10, wherein the cell is a cardiac cell.

16. The method of claim 10, wherein the cell is *in vivo*.

17. The method of claim 10, wherein the cell is *ex vivo*.

18. The method of claim 10, wherein the nonhypertension-associated cardiac disorder is selected from the group consisting of cardiac hypertrophy, nonhypertension-associated cardiac disease, heart failure after cardiac surgery, cardiac injury after myocardial infarction, myocardial ischemia, congestive heart failure and restenosis following angioplasty.

19. A method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension-associated renal disorder comprising

administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating a nonhypertension-associated renal disorder.

20. The method of claim 19, wherein the nucleic acid is administered to the subject in a virus.

21. The method of claim 20, wherein the virus is selected from the group consisting of adenovirus, retrovirus and adeno-associated virus.

22. The method of claim 20, wherein the virus is adenovirus.

23. The method of claim 19, wherein the nucleic acid is administered to the subject in a liposome.

24. The method of claim 19, wherein the cell is a kidney cell.

25. The method of claim 19, wherein the cell is *in vivo*.

26. The method of claim 19, wherein the cell is *ex vivo*.

27. The method of claim 19, wherein the nonhypertension-associated renal disorder is selected from the group consisting of renal injury, nephrotoxicity, nonhypertension-associated renal disease; salt-induced renal damage, glomerulosclerotic lesions, tubular injury, drug-induced renal damage, chronic renal failure, acute renal failure, nephrotic syndrome and diabetic nephropathy.

28. A method for treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension associated cardiac disorder, comprising administering to the subject a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding atrial natriuretic peptide is

expressed in a cell in the subject, thereby treating the nonhypertension-associated cardiac disorder.

29. The method of claim 28, wherein the nucleic acid is administered to the subject in a virus.

30. The method of claim 29, wherein the virus is selected from the group consisting of adenovirus, retrovirus and adeno-associated virus.

31. The method of claim 29, wherein the virus is adenovirus.

32. The method of claim 28, wherein the nucleic acid is administered to the subject in a liposome.

33. The method of claim 28, wherein the cell is a cardiac cell.

34. The method of claim 28, wherein the cell is *in vivo*.

35. The method of claim 28, wherein the cell is *ex vivo*.

36. The method of claim 28, wherein the nonhypertension-associated cardiac disorder is selected from the group consisting of cardiac hypertrophy, nonhypertension-associated cardiac disease, heart failure after cardiac surgery, cardiac injury after myocardial infarction, myocardial ischemia, congestive heart failure and restenosis following angioplasty.

37. A nucleic acid comprising an isolated nucleic acid encoding tissue kallikrein and an isolated nucleic acid encoding atrial natriuretic peptide.

38. The nucleic acid of claim 37 within an expression vector.

39. The nucleic acid of claim 38, wherein the expression vector comprises viral nucleic acid.

40. The nucleic acid of claim 39, wherein the viral nucleic acid is nucleic acid selected from the group consisting of adenovirus, retrovirus and adeno associated virus nucleic acid.

41. The nucleic acid of claim 37 in a liposome.

42. The nucleic acid of claim 37 in an adenoviral nucleic acid, wherein the nucleic acids can be packaged in an adenovirus particle and wherein expression of the nucleic acid encoding the tissue kallikrein and the nucleic acid encoding the atrial natriuretic peptide results in production of tissue kallikrein and atrial natriuretic peptide.

43. An adenovirus comprising the nucleic acid of claim 42.

44. A composition comprising a vector comprising a nucleic acid encoding tissue kallikrein and a vector encoding atrial natriuretic peptide.

45. The composition of claim 44, wherein the vector comprises viral nucleic acid.

46. The composition of claim 45, wherein the viral nucleic acid is nucleic acid selected from the group consisting of adenovirus, retrovirus and adeno-associated virus nucleic acid.

47. A method for delivering tissue kallikrein and atrial natriuretic peptide to a cell comprising administering to the cell a nucleic acid encoding tissue kallikrein and atrial natriuretic peptide under conditions whereby the nucleic acid is expressed, thereby delivering tissue kallikrein and atrial natriuretic peptide to the cell.

48. The method of claim 47, wherein the tissue kallikrein and atrial natriuretic peptide are delivered to the cell *in vivo*.

49. The method of claim 47, wherein the tissue kallikrein and atrial natriuretic peptide are delivered to the cell *ex vivo*.

50. The method of claim 47, wherein the nucleic acid is administered to the cell in a virus.

51. The method of claim 50, wherein the virus is selected from the group consisting of adenovirus, retrovirus and adeno-associated virus.

52. The method of claim 50, wherein the nucleic acid is administered to the cell in an adenovirus.

53. The method of claim 47, wherein the cell is selected from the group consisting of a heart cell, kidney cell, liver cell, lung cell, adrenal gland cell, endothelial cell, neuronal cell, myoblast and hematopoietic stem cell.

54. The method of claim 47, wherein the nucleic acid is administered to the cell in a liposome.

55. A method for treating a nonhypertension-associated renal disorder in a subject having a nonhypertension-associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the nonhypertension-associated renal disorder.

56. The method of claim 55, wherein the nucleic acids are administered to the subject in a virus.

57. The method of claim 56, wherein the virus is selected from the group consisting of adenovirus, retrovirus and adeno-associated virus.

58. The method of claim 56, wherein the virus is adenovirus.

59. The method of claim 55, wherein the nucleic acids are administered to the subject in a liposome.

60. The method of claim 55, wherein the cell is a kidney cell.

61. The method of claim 55, wherein the cell is *in vivo*.

62. The method of claim 55, wherein the cell is *ex vivo*.

63. The method of claim 55, wherein the nonhypertension-associated renal disorder is selected from the group consisting of renal injury, nephrotoxicity, nonhypertension-associated renal disease; salt-induced renal damage, glomerulosclerotic lesions, tubular injury, drug-induced renal damage, chronic renal failure, acute renal failure, nephrotic syndrome and diabetic nephropathy.

64. A method of treating a nonhypertension-associated cardiac disorder in a subject having a nonhypertension-associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and the nucleic acid encoding atrial natriuretic peptide are expressed in a cell in the subject, thereby treating the nonhypertension-associated cardiac disorder.

65. The method of claim 64, wherein the nucleic acids are administered to the subject in a virus.

66. The method of claim 65, wherein the virus is selected from the group consisting of adenovirus, retrovirus and adeno associated virus.

67. The method of claim 65, wherein the virus is adenovirus.

68. The method of claim 64, wherein the nucleic acids are administered to the subject in a liposome.

69. The method of claim 64, wherein the cell is a cardiac cell.

70. The method of claim 64, wherein the cell is *in vivo*.

71. The method of claim 64, wherein the cell is *ex vivo*.

72. The method of claim 64, wherein the nonhypertension-associated cardiac disorder is selected from the group consisting of cardiac hypertrophy, nonhypertension-associated cardiac disease, heart failure after cardiac surgery, cardiac injury after myocardial infarction, congestive heart failure and restenosis following angioplasty.

73. A method for treating and/or preventing a cerebrovascular disorder in a subject having a cerebrovascular disorder and/or at risk of having a cerebrovascular disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing the cerebrovascular disorder.

74. A method for treating and/or preventing an occlusive artery disorder (e.g., restenosis) in a subject having an occlusive artery disorder and/or at risk of having an occlusive artery disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic

peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing the occlusive artery disorder.

75. A method for inhibiting vascular smooth muscle cell growth and/or inhibiting neointimal formation in a blood vessel of a subject in need of inhibition of vascular smooth muscle cell growth and/or neointimal formation in a blood vessel, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby inhibiting vascular smooth muscle cell growth and/or neointimal formation in a blood vessel of the subject.

76. A method for treating and/or preventing renal damage and/or renal injury caused by drug-induced and/or salt-induced nephrotoxicity in a subject having renal damage and/or renal injury caused by drug-induced and/or salt induced nephrotoxicity and/or at risk of having renal damage and/or renal injury caused by drug-induced and/or salt-induced nephrotoxicity, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing renal damage and/or renal injury caused by drug-induced and/or salt-induced nephrotoxicity.

77. A method for stimulating renal tubular regeneration and/or reversing pre-existing renal injury in a subject in need of stimulation of renal tubular regeneration and/or reversal of pre-existing renal injury, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic

peptide is expressed in a cell of the subject, thereby stimulating renal tubular regeneration and/or reversing pre-existing renal injury in the subject.

78. A method for treating and/or preventing chronic renal failure in a subject having chronic renal failure or at risk of having chronic renal failure, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell of the subject, thereby treating and/or preventing chronic renal failure in the subject.

SEQUENCE LISTING

<110> MUSC Foundation for Research Development

Chao, Lee
Chao, Julie

<120> METHODS AND COMPOSITIONS FOR TREATING CARDIAC AND RENAL DISORDERS WITH ATRIAL NATRIURETIC PEPTIDE AND TISSUE KALLIKREIN GENE THERAPY

<130> 19113.0065/P

<150> 60/058,511
<151> 1997-09-11

<160> 30

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 1
cttcacataa gacagca 17

<210> 2
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 2
aacacagccc agtttgt 17

<210> 3
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 3
gacctcaaaa tcctgcc 17

<210> 4
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 4
gccacaaggg acgtagc 17

<210> 5
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 5
catttcagca ctttcca 17

<210> 6
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 6
acgaccttca cagcgtc 17

<210> 7
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 7
cactgagcac ttgtggg 17

<210> 8
<211> 20

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 8
caccgtgagc ttcctccttt 20

<210> 9
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 9
taggtcagac cagagct 17

<210> 10
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 10
tactacttcg gcgaatacct a 21

<210> 11
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 11
tccaatccgt caggtgtgat g 21

<210> 12
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:

/note="synthetic construct"

<400> 12
gacgacctgg ggacgact 18

<210> 13
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 13
aagacagcag tcaccact 18

<210> 14
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 14
ccgatctggt gtttgtc 17

<210> 15
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 15
aagactggga cctgctgtat 20

<210> 16
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 16
ccgtctggac ctccttgaac 20

<210> 17
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 17
gctgaggaca aagatgtt 18

<210> 18
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 18
tactccttca tggtccggaa cacc 24

<210> 19
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 19
gccacccagc tctgcaatat 20

<210> 20
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 20
ctgcccttgt catcacatga 20

<210> 21
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 21
tgtcacggtt gaagctt 17

<210> 22
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 22
gccacccagc tctgcaatat 20

<210> 23
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 23
ctgcccttgt catcacatga 20

<210> 24
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 24
ttcttgtact cgcacca 17

<210> 25
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 25

catttcagca ctttcca 17

<210> 26
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 26
gccacaaggg acgtagc 17

<210> 27
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 27
acgaccttca cagcgtc 17

<210> 28
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 28
aacacagccc agtttgt 17

<210> 29
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 29
cttcacataa gacagca 17

<210> 30
<211> 17
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of artificial sequence:
/note="synthetic construct"

<400> 30
gacctcaaaa tcctgcc 17

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00	A3	(11) International Publication Number: WO 99/12576
		(43) International Publication Date: 18 March 1999 (18.03.99)

(21) International Application Number: PCT/US98/19267

(22) International Filing Date: 11 September 1998 (11.09.98)

(30) Priority Data:
60/058,511 11 September 1997 (11.09.97) US

(71) Applicant *(for all designated States except US)*: MUSC FOUNDATION FOR RESEARCH DEVELOPMENT [US/US]; Suite 305, 141 Musc Complex, Cannon Park Place, Charleston, SC 29425 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CHAO, Lee [US/US]; 1011 Scottland Drive, Mt. Pleasant, SC 29464 (US). CHAO, Julie [US/US]; 1011 Scottland Drive, Mt. Pleasant, SC 29464 (US).

(74) Agents: MILLER, Mary, L. et al.; Needle & Rosenberg, P.C., 127 Peachtree Street, N.E., Atlanta, GA 30303 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
3 June 1999 (03.06.99)

(54) Title: METHODS AND COMPOSITIONS FOR TREATING CARDIAC AND RENAL DISORDERS WITH ATRIAL NATRIURETIC PEPTIDE AND TISSUE KALLIKREIN GENE THERAPY

(57) Abstract

The present invention provides a method for treating a nonhypertension–associated renal disorder in a subject having a nonhypertension associated renal disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating a nonhypertension–associated renal disorder. Also provided is a method of treating a nonhypertension–associated cardiac disorder in a subject having a nonhypertension–associated cardiac disorder, comprising administering to the subject a nucleic acid encoding tissue kallikrein and/or a nucleic acid encoding atrial natriuretic peptide under conditions whereby the nucleic acid encoding tissue kallikrein and/or the nucleic acid encoding atrial natriuretic peptide is expressed in a cell in the subject, thereby treating the nonhypertension–associated cardiac disorder.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/US 98/19267

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	J. CHAO ET AL: "Human Kallikrein Gene Delivery Attenuates Hypertension, Cardiac Hypertrophy , and Renal Injury in Dahl-Salt-Sensitive Rats" HUMAN GENE THERAPY, vol. 9, 1 January 1998, pages 21-31, XP002099183 cited in the application see abstract --- -/--	1-19, 38-78

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 April 1999	23/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Siatou, E

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/US 98/19267

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A,P	KUEI-FU LIN ET AL: "Atrial Natriuretic Peptide Gene Delivery Attenuates Hypertension, Cardiac Hypertrophy, and Renal Injury in Salt-Sensitive Rats" HUMAN GENE THERAPY, vol. 9, 1 July 1998, pages 1429-1438, XP002099184 cited in the application see abstract ---	20-78
A,P	H. MURAKAMI ET AL: " Human kallikrein gene delivery protects against gentamycin-induced nephrotoxicity in rats" KIDNEY INTERNATIONAL, vol. 53, 1998, pages 1305-1313, XP002099190 see abstract ---	1-19, 38-78
A,P	L. JIN ET AL: "Gene Therapy in Hypertension: Adenovirus-Mediated Kallikrein Gene Delivery in Hypertensive Rats" HUMAN GENE THERAPY, vol. 8, 10 October 1997, pages 1753-1761, XP002099185 see abstract ---	1-19, 38-78
A	J. CHAO ET AL: "Experimental Kallikrein Gene Therapy in Hypertension, Cardiovascular and Renal Diseases" PHARMACOLOGICAL RESEARCH, vol. 35, no. 6, 1997, pages 517-522, XP002099186 see abstract see page 519, right-hand column, last paragraph - page 521, left-hand column ---	1-19, 38-79
A	KUEI-FU LIN ET AL: "Human Atrial Natriuretic Peptide Gene Delivery Reduces Blood Pressure in Hypertensive Rats" HYPERTENSION, vol. 26, no. 6(1), December 1995, pages 847-853, XP002099187 cited in the application see abstract ---	20-78
A	PATENT ABSTRACTS OF JAPAN vol. 5, no. 132 (C-068), 22 August 1981 & JP 56 068618 A (GREEN CROSS CORP), 9 June 1981 see abstract --- -/--	1-19

2

INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/US 98/19267

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	H. S. MARGOLIUS : "Tissue Kallikreins and Kinins: Regulation and Roles in Hypertensive and Diabetic Diseases" ANNUAL REVIEW OF PHARMACOLOGY AND TOXICOLOGY. vol. 29, 1989, pages 343-364, XP002099188 cited in the application see page 355, line 1 - page 356, last paragraph	1-78

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 19267

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-78 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.